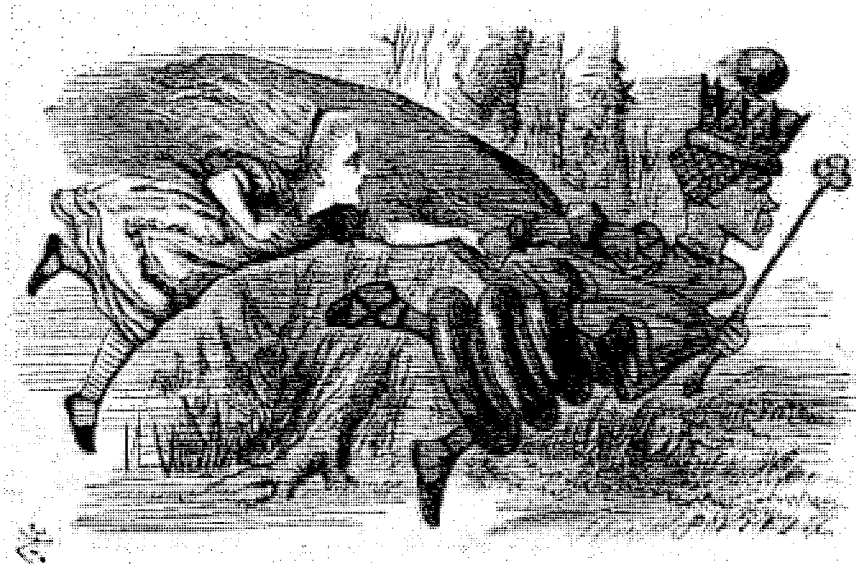


**Long live the Red Queen? Examining environmental influences on host-parasite interactions in *Daphnia*.**



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## Abstract

The Red Queen hypothesis proposes that antagonistic coevolution between parasites and their hosts is responsible for the evolutionary maintenance of sexual reproduction. It suggests that frequency-dependent selection by parasites against common host genotypes prevents asexual clones capitalising on their two-fold reproductive advantage and out-competing their sexual counterparts. However, in order for the Red Queen to be effective parasites must be highly virulent and display genotype-specific infection patterns, and both of these requirements are known to be affected by environmental conditions. In this thesis I examine environmental influences over host-parasite interactions in *Daphnia*.

A survey of parasite prevalence in North American populations of *Daphnia pulex* represents the first attempt to examine the role of parasites in the maintenance of breeding system variation in this species. Despite evidence of over- and underparasitism in some populations, parasite prevalences were generally very low suggesting that parasites are not a major source of selection in the populations studied.

The Pluralist Approach to sex proposes that the effects of deleterious mutations and parasitism may interact. I established mutant lines of *Daphnia magna* using the chemical mutagen ENU and investigated the impact of the parasite *Pasteuria ramosa* on mutation load under different environmental conditions. I found that although parasite infection could exacerbate the effects of mutation load, this interaction was dependent on host environment and the implications of these findings for the general application of the Pluralist Approach are discussed.

The impact of mixed strain infections on genotype-specific infection was also examined. In natural populations, hosts are likely to be exposed to a range of parasite genotypes and this may potentially affect the efficiency of the immune response. I found that the ability of certain *P. ramosa* strains to infect their hosts is affected by prior host exposure to different strains.

“If the Red Queen is not the answer, let us see her flee from the attack of more solid data....”

- Curt Lively, June 2005

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# Chapter 1. General Introduction

## 1.1. Summary

The Red Queen hypothesis proposes that antagonistic coevolution between parasites and their hosts is responsible for the evolutionary maintenance of sexual reproduction. It suggests that frequency-dependent selection by parasites against common host genotypes prevents asexual clones capitalising on their two-fold reproductive advantage and out-competing their sexual counterparts. However, in order for the Red Queen to be effective parasites must be highly virulent and display genotype-specific infection patterns, and both of these requirements are known to be affected by environmental conditions. In this thesis I examine various aspects of the Red Queen hypothesis using the Cladoceran crustacean *Daphnia*.

In Chapter 2, I examine patterns of parasite prevalence and breeding system variation across twenty-one *Daphnia pulex* populations in southwest Ontario, Canada. Obligate parthenogenesis in this species is estimated to have arisen between 170-300,000 years ago (Paland *et al.*, 2005), and asexuals have come to dominate most of northeast North America. However, sexual populations still exist in some areas and it is not clear how these pockets of sexuality are being maintained. The work described in this chapter represents the first attempt to examine the potential role of parasites in the maintenance of breeding system variation in this species.

In Chapter 3, I test the pluralist approach to sex (West *et al.*, 1999) by looking at interactions between deleterious mutation load and parasitism, using *Daphnia magna* and

the bacterial endoparasite *Pasteuria ramosa*. The pluralist approach proposes that the effects of parasitism and mutation load may have to act synergistically in order to stabilise sex, and one of the ways this could occur is if parasites can cause synergistic epistasis between deleterious mutations in their hosts. Although previous studies have found little evidence to support this hypothesis (Peters, 1999; Salathé & Ebert, 2003; Haag *et al.*, 2003; Cooper *et al.*, 2005), they have failed to take into account the fact that the effects of mutation load and parasitism can often vary in different environments. Here I examine the effects of mutation load and parasitism using different parasite genotypes, under a range of environmental conditions.

Finally, in Chapter 4, I investigate the specificity underlying patterns of resistance and virulence in the *D. magna* – *P. ramosa* system. There is evidence of strong parasite genotype-by-host genotype interactions in this system (Carius *et al.*, 2001), but the mechanisms underlying this specificity are not well understood. By consecutively exposing *D. magna* lines to different *P. ramosa* strains, I aim to examine the specificity of the immune response in *D. magna*.

## **1.2. The puzzle of sex and recombination**

Explaining the evolution of sexual reproduction remains one of the greatest challenges facing evolutionary biology. Not only is it unclear how this rather uneconomical form of reproduction originated, its continued persistence – thought to be in excess of one billion years (Otto & Lenormand, 2002) – and almost ubiquitous occurrence amongst Eukaryotic taxa has proved something of an enigma to biologists for many years (Bell, 1982).

The costs of sex are many and varied. Not only do many sexually-reproducing organisms incur the energetic costs associated with finding and securing a mate, the accompanying process of genetic recombination (the process by which DNA is exchanged between homologous chromosomes during meiosis) also has the potential to destroy any gene combinations that may have been considered favourable during the parental generation (Otto & Lenormand, 2002). If an organism's genome has seen it through to reproductive age then mixing it up with the genome of another individual seems nonsensical, and this especially holds true if it results in the production of offspring with reduced fitness (known as the 'recombination load') (Charlesworth & Barton, 1996).

Perhaps the most pressing problem faced by sexual organisms is what is commonly referred to as the 'two-fold cost of sex', or the 'cost of males'. Working on an 'all else being equal' assumption, a sexual population adhering to Fisher's predicted 1:1 sex-ratio (Fisher, 1930) will only be half as productive as an asexual one producing exclusively female progeny. From an evolutionary point of view this difference is huge and places sexuals at a significant disadvantage; theory predicts that such a disadvantage would see a sexual population driven to extinction by an asexual clone in around 50 generations (Wuethrich, 1998).

Add all of this to the fact that sexually reproducing organisms also leave themselves vulnerable to exploitation by selfish cytoplasmic elements (Hastings, 1999), sexually-transmitted diseases (Nunn *et al.*, 2000) and the potential evolution of intersexual conflict (Holland & Rice, 1999), and we are faced with something of a dilemma.

### **1.3. Parasites, deleterious mutations and the evolution of sex**

The hypotheses put forward to explain sex are generally categorised as being either ecological or genetic (Rice, 2002). From these groupings there are two hypotheses in particular that are accepted as being the most plausible, and this is largely due to their general applicability; the majority of the hypotheses put forward tend to make restrictive assumptions or only really apply to particular groups of organisms (Bell, 1982; Kondrashov, 1988, 1993; Hamilton *et al.*, 1990; Barton & Charlesworth, 1998; Wuethrich, 1998; West *et al.*, 1999). The Parasite Red Queen hypothesis (ecological) deals with antagonistic coevolution between parasites and their hosts, whereas the Mutational Deterministic hypothesis (genetic) suggests that sex acts to aid in the purging of deleterious mutations from the genome.

#### **1.3.1. Sex and parasites**

As was briefly discussed above, recombination during meiosis has the ability to break apart gene combinations that may have been favoured in previous generations. When such gene combinations are overrepresented in a population they are said to be in positive linkage disequilibrium and this may result when there is positive epistasis between the loci involved (that is, the interaction between said loci leads to disproportionately high fitness) (Peters & Lively, 1999). Sex and recombination are unlikely to be favoured in

such a situation, as most offspring produced are likely to suffer from reduced fitness (Charlesworth & Barton, 1996; Otto & Lenormand, 2002). However, Barton (1995) has predicted that recombination may be selected for if the sign of epistasis fluctuates over very short periods of time. That is, a combination of alleles that is advantageous in one generation must become disadvantageous (and vice-versa) in the space of two to five generations if recombination is to be favoured. It has for some time been suggested that genotype-specific frequency-dependent selection by parasites may play an important role in the evolutionary maintenance of sex (Jaenike, 1978; Hamilton, 1980; Hamilton *et al.*, 1990) and recent theoretical work has shown that antagonistic coevolution between parasites and their hosts may be able to cause fluctuating epistasis over the appropriate timescale (Peters & Lively, 1999).

It is generally assumed that parasites will be selected to infect the most locally common host genotype (Hamilton *et al.* 1990) and under these circumstances common genotypes may find themselves becoming disproportionately infected. This will result in the sign of epistasis between loci (in this case resistance loci) changing from positive to negative (Peters & Lively, 1999). Frequency-dependent selection acting in this manner may prevent an asexual clone rapidly increasing in frequency and overrunning a sexual population, as any clone that becomes too common will be driven down by parasites. One of the main effects of recombination in sexual populations will be to break up positive linkage disequilibria and generate genotypes that are relatively rare in the population. Due to the fact that these rare genotypes are less likely to become infected, epistasis between the loci involved will be positive. Therefore under fluctuating epistasis

recombination will provide the biggest advantage when linkage disequilibrium and epistasis are of opposite signs (Barton, 1995; Peters & Lively, 1999).

The concept described above is commonly referred to as the Parasite Red Queen (PRQ) hypothesis, and of all the ecological hypotheses it is certainly the one that has received the most attention. A review of empirical work on the subject shows that most studies attempt to test the predictions of the PRQ by either (a) examining the relationship between host genotype frequency and infection by parasites (e.g. Lively *et al.*, 1990; Kelley, 1994; Vernon *et al.*, 1996; Dybdahl & Lively, 1998; Little & Ebert, 1999), (b) comparing levels of infection in closely related sexual and asexual species (e.g. Moritz *et al.*, 1991; Hanley *et al.*, 1995; Hakoyama, 2001; Michiels *et al.*, 2001; Kumpulainen *et al.*, 2004), or (c) looking for a relationship between levels of infection and levels of sex and recombination (e.g. Lively, 1987; Heller & Farstey, 1990; Jokela & Lively, 1995; Camacho *et al.*, 2002; Ben-Ami & Heller, 2005). Those in the first category are undoubtedly the most informative, as the prediction that parasites attack genotypes in a frequency-dependent manner lies at the very heart of the PRQ. Studies that fall into the second category may have their merits but in general they are of limited value, as they rarely tend to examine the underlying dynamics. Simply showing that asexual lineages are more prone to parasitic infection does not necessarily support the PRQ *per se*, as unless it can be shown that susceptibility is due to genotype-specific interactions it is not possible to rule out alternative explanations, such as reductions in fitness due to accumulation of deleterious mutations, possible effects of polyploidy in hybrids etc. (Ladle, 1992; Brown *et al.*, 1995; Michiels *et al.*, 2001). The final category deals with the prediction that if the variance in risk and prevalence of infection is high then there

will be a positive correlation between levels of prevalence and levels of sex; sex will be favoured (or more importantly, asex will be disfavoured) in areas with high prevalence, whereas in areas of low prevalence asexual reproduction is predicted to be more common (Lively, 2001). Ideally, PRQ studies should attempt to incorporate all three approaches, though to date this has only been achieved for one model system (Jokela *et al.*, 2003).

Despite being the most popular of the ecological hypotheses, the PRQ has still been criticised on several counts. For example, in order to work most effectively it tends to require high parasite virulence and /or high parasite transmission rates, which narrows the range of conditions under which it can select for sex (Howard & Lively, 1994). It has also been pointed out that frequent mutation to parthenogenesis by individuals from the sexual population will eventually erode the advantage of sex; as more and more clones become established the level of genetic diversity in the asexual population will increase, hence diminishing any advantage provided by recombination (Howard & Lively, 1994).

### **1.3.2. Sex and deleterious mutations**

Copying errors during the processes of DNA replication and repair are almost inevitable (Ridley, 1996). Although mutation ultimately provides the genetic variation necessary for evolution to proceed, the majority of non-neutral genetic mutations are likely to be deleterious (Kondrashov, 1988). It will therefore be in the best interests of most organisms to prevent their accumulation in the genome.

Two main hypotheses deal with the role of mutation accumulation in the evolution of sex. The first, Muller's Ratchet, deals with the accumulation of mutations through stochastic processes and suggests that the main advantage of sex is its ability to reconstitute genotypes bearing very few, or even zero, mutations (Kondrashov, 1993). Within an asexual population (or even within an asexual lineage) there is likely to be variation between individuals in the number of deleterious mutations they are carrying, with one 'optimal' class carrying no (or very few) mutations. Should this optimal class be lost from the population through stochastic processes then the Ratchet clicks round one notch and, in the absence of back mutation, it cannot be recovered (Kondrashov, 1993). Rice (1998, 2002) has shown that if at any point the excess in the net reproductive rate of the fittest class is unable to compensate for the loss from that class by mutation (known as the requisite mutational load), then mutations will start to accumulate in a deterministic manner. If the accumulation of mutations in this manner leads to a decrease in population size then the population will eventually go into 'mutational meltdown' (Lynch *et al.*, 1993).

Of course, if recombination is capable of generating genotypes bearing low numbers of mutations then it can also generate genotypes carrying many. According to the Mutational Deterministic hypothesis (MDH), if these 'bad' genotypes fail to reproduce then the mutations they carry will be purged from the population (Kondrashov, 1988). Therefore sex has the ability to increase the variance in offspring fitness, creating very fit genotypes with high productivity and very unfit ones with little, or no, productivity. In contrast, the fitness variance of asexually-produced offspring will be relatively small and asexual lineages will not be able to purge large numbers of mutations



in the way sexual ones can (Kondrashov, 1993). Like the PRQ this hypothesis relies on recombination's ability to break up linkage disequilibria, though in this case it is in response to directional, rather than fluctuating, selection. Negative epistasis between alleles will lead to negative linkage disequilibrium, which in turn will reduce the additive genetic variance in fitness (Barton, 1995). The effect of recombination then is to break up linkage disequilibria and bring these alleles together, thus allowing selection to act upon them more efficiently.

Although in theory the MDH has been shown to compensate for the twofold cost of sex, it can only do so if certain criteria are met; like the PRQ, the MDH relies on several specific assumptions in order to render sex stable. In this case the requirements are that the rate of deleterious mutation per genome per generation ( $U$ ) is equal to or greater than one, and that there is synergistic epistasis between deleterious mutations (Kondrashov, 1988). Synergistic epistasis is said to occur when the addition of a mutation leads to a greater decrease in log fitness than the last, i.e. there is a nonlinear decrease in log fitness as mutations accumulate (West *et al.*, 1998). Unfortunately, there is still little evidence that either of these requirements can be satisfied. Estimates of  $U$  vary widely, both within and between species (Drake *et al.*, 1998; Lynch *et al.*, 1999; Keightley & Eyre-Walker, 2000), and it has even been shown that there may be a positive linear relationship between mutation rate and generation time; if this is the case then the MDH may not apply to species with short generation times (Keightley & Eyre-Walker, 2000). Disagreement over the methods used to calculate  $U$  has not helped the issue (Keightley & Eyre-Walker, 2001; Kondrashov, 2001), and this has been exacerbated by empirical work

showing that the vast majority of deleterious mutations (>90%) may be cryptic (Davies *et al.*, 1999).

The subject of how deleterious mutations interact also remains controversial. While some studies have found evidence for negative synergistic interactions (Mukai, 1969; de Visser *et al.*, 1996; Whitlock & Bourguet, 2000; Rivero *et al.*, 2003), others have found no evidence for synergism (Elena & Lenski, 1997; de Visser *et al.*, 1997; Pena *et al.*, 2000; Wloch *et al.*, 2001) and at least one has shown that the type of epistasis displayed may depend on the sort of environment the organism is tested in; an *in silico* study by You and Yin (2002) found that mutations in bacteriophage T7 acted synergistically in poor-resource environments but antagonistically in high-resource environments. Several workers have suggested that synergistic interactions may only become apparent if organisms are tested under competitive conditions (Peck & Waxman, 2000; Rivero *et al.*, 2003), the main implication being that experiments attempting to demonstrate negative epistasis should be redesigned to make them more naturalistic (Peck & Waxman, 2000). The crossing methods used to test for epistasis in sexual species have also come under criticism from some authors (West *et al.*, 1998).

#### **1.4. Alternative hypotheses**

Over 20 different hypotheses have been put forward to explain the evolutionary advantage of sex (Kondrashov, 1993), though the majority of these tend to make

restrictive assumptions or really only apply to particular groups of organisms (Bell, 1982; Kondrashov, 1988, 1993; Hamilton *et al.*, 1990; Barton & Charlesworth, 1998; Wuethrich, 1998; West *et al.*, 1999). The Tangled Bank hypothesis, for example, proposes that the diversity produced through sex and recombination is beneficial in heterogeneous environments as it allows sexuals to exploit a wider range of resources, thus reducing competition between individuals (Bell, 1982). Asexual clones, in contrast, are expected to be genotypically similar and thus more likely to compete for similar resources. The main flaw in this hypothesis is the assumption that the asexual component of the population is lacking in genotypic diversity; like the Red Queen hypothesis, the advantage provided to sexuals by recombination is eroded if there is a significant degree of asexual genotypic diversity (Bell, 1982). It also requires a certain degree of environmental complexity, that the cost of sex is small and that competition between different genotypes is limited (Bell, 1982), making it an unlikely candidate as a general explanation for the evolutionary maintenance of sex.

One alternative hypothesis which has gained considerable favour over recent years, due mainly to the fact that it actually has some semblance of empirical support, suggests that sex is advantageous because it speeds up the rate at which beneficial mutations become fixed (Bell, 1982; Burt, 2000; Colegrave; 2002). The underlying mechanism here is similar to that of the Mutational Deterministic hypothesis, in that it relies upon recombination's ability to break up particular gene combinations and create new ones, but in contrast to the MDH the advantage comes from combining beneficial, not deleterious, mutations (Bell, 1982). For example, if two new beneficial mutations arise in separate lineages within an asexual population the only way in which they can be

brought together within a single lineage is if they either both arise simultaneously within the same lineage, or if they occur successively within a lineage. This latter scenario, of course, depends on the rate at which the initial mutations sweep through the population. If both mutations arise simultaneously in separate lineages, there is also the possibility that clonal interference will slow the rate at which the mutations become fixed (Colegrave, 2002; De Visser & Rozen, 2006). In contrast, in a sexual population the processes of recombination and sexual reproduction allow new beneficial mutations to be brought together relatively quickly (Colegrave, 2002). This concept has been expanded further to address the potentially negative effect deleterious mutations may have on newly arising beneficial mutations. For example, 'background trapping' may occur if a beneficial mutation arises in a genome bearing a large number of deleterious mutations; under such circumstances, if the positive effect of the beneficial mutation does not outweigh the negative effect of the deleterious mutations already present, there is a strong possibility that the new mutation will be lost before it has a chance to spread (Peck, 1994; Hadany & Feldman, 2005). In sexual populations this is less likely to be a problem, as the beneficial mutation can be freed from the negative impact of deleterious mutations and find itself on a more favourable genetic background (Peck, 1994). On the other hand, if the positive effect of the beneficial mutation is greater than the negative effect of the deleterious mutations already present, the new mutation may spread but drag all of the deleterious mutations along with it. This process of 'evolutionary traction' (Hadany & Feldman, 2005) may considerably slow down the rate at which adaptation occurs, as deleterious mutations will continually accumulate as new beneficial mutations are

selected for. Again, the advantage to sex under these circumstances arises from its ability to free-up beneficial mutations from genetically poor backgrounds.

### **1.5. Pluralism: breaking the deadlock?**

Even though both parasites and deleterious mutations are likely to affect the vast majority of living things, it has so far been difficult to show that either one of them is solely responsible for the evolutionary maintenance of sex. This is in part due to the fact that the above theories make explicit assumptions about various parameters and if those assumptions are not met then neither model is able to fully compensate for the cost of sex.

In response to this dilemma, some authors have pointed out that viewing the individual theories as being mutually exclusive may actually be counterproductive, as the different mechanisms could potentially combine to cover each other's weaknesses (West *et al.*, 1999). Howard and Lively (1994) were the first to address this idea formally when they demonstrated that a combination of host-parasite coevolution and mutation accumulation through Muller's ratchet could make sex stable. This occurred even when the main parameter values (i.e. mutation rate, parasite virulence and parasite transmission probability) were lower than each theory would normally require in isolation. The two mechanisms can combine to work as follows: If an asexual mutant pops up in a sexual population it is likely to spread fairly rapidly thanks to its two-fold reproductive

advantage, coupled with the fact that is also expected to be carrying fewer than the equilibrium number of deleterious mutations (Howard & Lively, 1998). However, as soon as the clone starts to become increasingly common it is likely to attract the attention of evolving parasites. These will be selected to disproportionately infect any genotype that becomes overrepresented in the population and as a result the clone will be driven down in frequency. Each time the clone has its numbers reduced in this way it will become more susceptible to mutation accumulation by Muller's ratchet, either through 'stochastic elimination' (the chance loss of all individuals from the least-loaded class) or 'stochastic loading' (the fewer individuals there are in the least-loaded class, the more likely it is that they will all receive at least one additional mutation) (Howard & Lively, 1998). Parasites may therefore work to protect sex in the short term, whereas mutation accumulation acts to drive clones to extinction in the long term.

Of course, there are other ways in which mutations and parasites could interact. For example, parasites may be directly responsible for causing synergistic epistasis between deleterious mutations; under circumstances in which mutations normally have independent (multiplicative) effects the addition of a pathogen may change the shape of the mutation-fitness curve (Peters, 1999). As has been discussed above, synergistic interactions may only become apparent once organisms have been placed under highly selective conditions (e.g. low-resource environments, competitive situations etc.), and parasitic infections can almost certainly be considered to be a source of strong selection. Alternatively, the accumulation of deleterious mutations may reduce the fitness of a genetic lineage in such a way that it becomes more susceptible to parasitic infections in general. This could include pathogens that do not normally attack in a genotype-specific

manner (e.g. opportunistic bacterial pathogens), so it could occur even in the absence of Red Queen-type dynamics.

## **1.6. *Daphnia*: Model system for studying the evolution of sex and recombination**

The 50+ species in the genus *Daphnia* form a major part of freshwater zooplankton communities worldwide (Hebert, 1978). Their widespread occurrence, mode of reproduction and amenability as experimental organisms in both the laboratory and the field has made them popular subjects for studies on population genetics, host-parasite coevolution and the evolution of reproductive strategies (e.g. Hebert, 1978; Lynch, 1984, 1987; Korpelainen, 1986; Ruvinsky *et al.*, 1986; Weider, 1993; Geedey *et al.*, 1996; Innes, 1997; Ebert *et al.*, 1998; Little & Ebert, 1999, 2001; Innes & Singleton, 2000; Innes *et al.*, 2000; Berg *et al.*, 2001; Carius *et al.*, 2001).

### **1.6.1. The *Daphnia* lifecycle**

The majority of *Daphnia* species are cyclically parthenogenetic, a mode of reproduction known in at least seven other invertebrate taxa (Hebert, 1987). During favourable conditions adult females reproduce through apomictic parthenogenesis; mitotically-produced diploid eggs are released from the ovaries and then develop in a brood chamber located on the dorsal side of the carapace. These eggs normally develop into females, though under the influence of certain environmental cues (e.g. changes in photoperiod,

food quality/quantity and crowding) they develop into males (Hebert, 1978; Berg *et al.*, 2001). Broods are usually single-sexed, although mixed broods are known to occur (Hebert, 1978). The same cues that lead to male development are also responsible for the initiation of sexual reproduction (Kleiven *et al.*, 1992; Berg *et al.*, 2001). When this occurs, females produce haploid eggs through meiosis and these normally have to be fertilised in order to develop. These eggs are produced in pairs and enclosed within an ephippium, a protective structure composed of several membranous layers (Hebert, 1978). Ephippial eggs are resistant to desiccation and freezing and constitute the resting stage of the *Daphnia* lifecycle, remaining in diapause until environmental conditions become more favourable (Hebert, 1978).

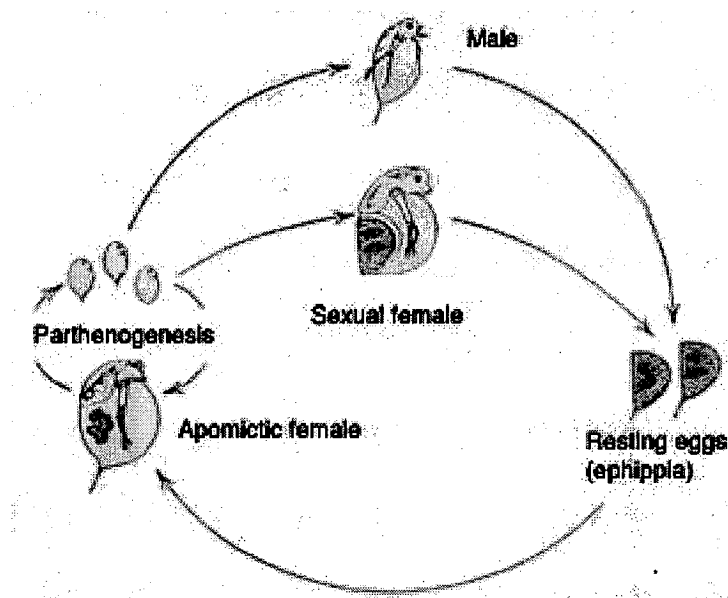


Figure 1.1. The *Daphnia* lifecycle. (Adapted from [www.o-fish.com](http://www.o-fish.com))



### **1.6.2. *Daphnia* in the laboratory**

The ability to manipulate the reproductive behaviour of *Daphnia* species in the laboratory makes them ideal for studying the evolution of reproductive strategies, including hypotheses put forward to explain the evolutionary maintenance of sex. Different clonal genotypes can easily be distinguished using allozyme electrophoresis (Hebert & Beaton, 1993) and by providing conditions that continually favour parthenogenetic reproduction it is possible to “freeze” these genotypes for as long as is required. The ability to propagate clones in this manner makes them ideal for mutation accumulation studies (an approach often used when estimating mutation rates or testing for epistatic interactions e.g. Mukai 1964, 1969; Shabalina *et al.*, 1997; Fry *et al.*, 1999; Lomnicki & Jasienski, 2000; Vassilieva *et al.*, 2000).

### **1.6.3. Obligate parthenogenesis in *Daphnia***

The occurrence of obligate parthenogenesis in several *Daphnia* species also presents the opportunity to study ecological hypotheses of sex (Hebert, 1981; Hebert & Crease, 1983). In contrast to cyclically parthenogenetic forms, obligate parthenogens produce ephippial eggs that are able to develop without being fertilised. Allozyme and breeding studies have shown that these eggs are diploid and ameiotically produced (Hebert, 1981; Innes & Hebert, 1988). Studies of North American *Daphnia pulex* populations have revealed extensive breeding system variation across the length and breadth of the continent (Hebert *et al.*, 1993; Hebert & Finston, 2001; Paland *et al.*, 2005), with asexual and

sexual populations often coexisting within the same geographical region. Genetic studies strongly suggest that obligate parthenogenesis in this species is due to the action of a meiotic-suppressor gene (Innes & Hebert, 1988). Intriguingly, meiosis is only suppressed in female carriers and because some obligate females are still capable of producing male progeny (Hebert & Crease, 1983) it has been possible to cross these males with cyclical females (Innes & Hebert, 1988). Innes and Hebert (1988) found that four out of ten hybrids produced in this way could reproduce by obligate parthenogenesis, which confirms the genetic basis of meiosis-suppression and also supports the idea that asexuality in this species may have spread in a 'contagious' manner (Simon *et al.*, 2003; Paland *et al.*, 2005).

However, the speed at which asexuality spreads may be reduced if clones do not automatically cease male production. Many parthenogenetic species are still capable of producing males (Simon *et al.*, 2003) and in such cases any potential reproductive advantage will be relatively low until male production (or allocation to male function) has been reduced. Such a reduction is likely to occur most readily in situations in which there is a significant cost to male production (e.g. Jokela *et al.*, 1997; Innes *et al.*, 2000).

#### **1.6.4. Parasitism in *Daphnia***

Natural *Daphnia* populations are affected by a wide range of macro- and microparasites (Green, 1974; Little & Ebert, 1999). The bacterial endoparasite *Pasteuria ramosa* is known to reach high prevalence in *D. magna* populations across Europe (Ebert *et al.*, 1998; Carius *et al.*, 2001), and infection studies have revealed genetic variation for both

resistance and virulence within these populations (Ebert *et al.*, 1998; Carius *et al.*, 2001; Little & Ebert, 2001). Transmission of *P. ramosa* is primarily horizontal, with infection occurring when waterborne spores are taken up by the filter-feeding host (Ebert *et al.*, 1996). Once established, the parasite grows to fill the entire body cavity of its host and infections almost always result in host sterilisation (Carius *et al.*, 2001). The death of the host is then necessary for the bacterial spores to be released (Ebert *et al.*, 1996; Carius *et al.*, 2001).

The nature of this particular host-parasite system makes it an ideal candidate for Red Queen studies. The existence of genotype-specific infection patterns opens up the potential for antagonistic coevolution and frequency-dependent selection (Little & Ebert, 1999; Carius *et al.*, 2001), and indeed there is good evidence that *P. ramosa* may be driving genotype frequency changes in natural *D. magna* populations (Little & Ebert, 2001). The consequences of *P. ramosa* infection are also very dire, which means that this parasite should be capable of providing the selective pressure required by Red Queen models (Hamilton *et al.*, 1990; Howard & Lively, 1994; West *et al.*, 1999).

## Chapter 2. Breeding system variation and parasite prevalence in North American *Daphnia pulex*.

### 2.1. Abstract

The Red Queen hypothesis proposes that frequency-dependent selection by parasites may be responsible for the evolutionary maintenance of sexual reproduction. I aimed to test several aspects of the Red Queen hypothesis by examining patterns of parasitism in obligately and cyclically parthenogenetic populations of *Daphnia pulex* (Crustacea; Cladocera) over a four-week period. Three different measures of sexuality were regressed against mean levels of parasite prevalence across twenty-one separate *D. pulex* populations, and contingency table analysis was used to look for evidence of frequency-dependent selection within populations.

Overall, levels of parasitism were low and no relationship was found between levels of sexuality and mean parasite prevalence. Evidence for frequency-dependent infection was detected in two out of the twenty-one populations, with several different clones showing signs of over- and underparasitism. However, the results suggest that parasites are not a major source of selection in these populations and as such it seems unlikely that they are responsible for maintaining breeding system variation across the study region.

## **2.2. Introduction**

### **2.2.1. *Parasites and sex***

The predominance of sexual reproduction amongst Eukaryotic taxa has long puzzled evolutionary biologists, as the ‘two-fold cost’ associated with male production should put sexual taxa at a major disadvantage when they come into competition with their asexual counterparts (Maynard Smith, 1978). This dilemma has received a lot of attention over the past few decades and as a result a large number of genetic and ecological hypotheses have been put forward in an attempt to explain both the long-term and short-term maintenance of sex (Bell, 1982; Kondrashov, 1993; West *et al.*, 1999).

One of the most prominent ecological explanations for sex, the Parasite Red Queen hypothesis, proposes that frequency-dependent selection by parasites is responsible for preventing the spread of asexuality (Bell, 1982). The Parasite Red Queen hypothesis works on the assumption that parasites will be selected to infect the commonest host genotypes (Hamilton *et al.*, 1990). In this respect, sex is thought to be advantageous due to recombination’s ability to create novel genotypes, thus limiting parasite adaptation to particular genotypic combinations. Consequently, in the longer term, sexual populations may have higher geometric mean fitness.

The hypothesis also makes several predictions as to how prevalence may vary between breeding systems depending on the distribution of parasite abundance. For example, if parasite abundance shows little variance across populations then sexuals are expected to bear lower parasite loads than their asexual counterparts. On the other hand, if the risk of infection varies widely across populations then asexuals are predicted to

dominate in low-risk areas whereas sexuals are expected to hold precedence in areas where parasites are more abundant (Lively, 2001).

The aim of this study was to investigate various aspects of the Red Queen hypothesis by examining patterns of parasitism in cyclically and obligately asexual populations of the Cladoceran crustacean *Daphnia pulex*. Specifically, I wanted to (a) compare levels of parasitism with levels of sex across populations, and (b) look for evidence of frequency-dependent infection patterns within infected populations.

### **2.2.2. Reproduction and parasitism in *Daphnia***

Like the majority of *Daphnia* species, most populations of *D. pulex* are cyclically parthenogenetic. During favourable periods adult females reproduce through apomixis, though in response to certain environmental cues they will instead start to produce pairs of haploid eggs through meiosis. These eggs require fertilisation in order to develop and, enclosed within a protective ephippium, they constitute the resting stage of the *Daphnia* lifecycle (Hebert, 1978).

The occurrence of obligate parthenogenesis in several *Daphnia* species has been recognised for some time (Hebert, 1981; Hebert & Crease, 1983), and studies of North American *Daphnia pulex* populations have revealed that asexual (obligately parthenogenetic) and sexual (cyclically parthenogenetic) populations often coexist within the same geographical region (Hebert *et al.*, 1989; Hebert *et al.*, 1993; Hebert & Finston, 2001). In contrast to cyclically parthenogenetic forms, obligate parthenogens produce ephippial eggs that develop without being fertilised, and genetic studies have revealed

that this is mainly due to the action of a sex-linked meiotic-suppressor gene (Innes & Hebert, 1988). Allozyme and breeding studies have confirmed that these eggs are diploid and ameiotically produced (Hebert, 1981; Innes & Hebert, 1988). This aspect of the species' biology is particularly appealing as it means that the potentially confounding factors of ploidy difference and/or hybridisation, common to other Red Queen study systems (e.g. Lively, 1990; Moritz *et al.*, 1991; Hanley *et al.*, 1995; Brown *et al.*, 1995; Hakoyama *et al.*, 2001; Michiels *et al.*, 2001), can be avoided.

*Daphnia* are attacked by a wide array of microparasites (Green, 1974; Stirnadel & Ebert, 1997), and many of these parasites are known to have substantial effects on host fitness (Green, 1974; Schwartz & Cameron, 1993; Ebert, 1995; Mangin *et al.*, 1995; Stirnadel & Ebert, 1997; Little, 1999). Parasite biomass within hosts is often considerable and, because *Daphnia* have a transparent carapace, it is possible to detect many infections without dissection. This study represents one of the first attempts to examine the role of parasitism in the evolution of breeding system in North American *Daphnia pulex*.

## **2.3. Materials and methods**

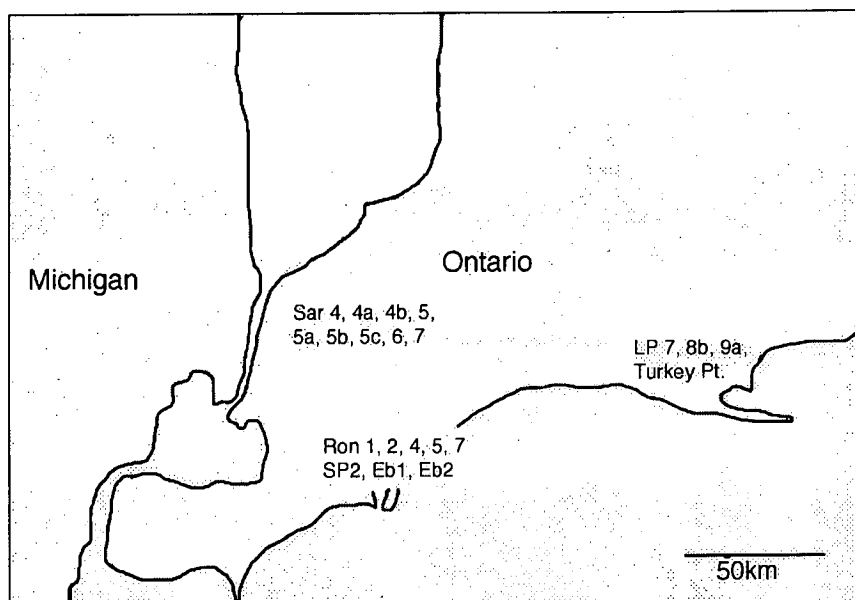
### **2.3.1. Sampling and examination**

Samples were collected from 21 ponds in southwest Ontario (Fig. 1) over a four-week period during the Spring of 2003. This geographic area is known to contain both sexual and obligately asexual populations (Hebert *et al.*, 1988; Hebert *et al.*, 1993). The

majority of populations were located in small, temporary melt-water ponds and as such the length of the sampling period was constrained by the ephemeral nature of these habitats. One sample was taken from each pond per week using a fine-meshed aquarium net, and samples were immediately cooled and maintained at between 1°C and 5°C to minimise mortality before inspection (always within 24 hours). After each sample was collected, sampling equipment was cleaned and sterilised using 70% ethanol to avoid spreading parasites and hosts between different ponds.

In the laboratory, an initial random sub-sample of ~100-600 individuals (depending on the density of the whole sample) was examined under a dissecting microscope. These initial sub-samples were used to estimate the sex-ratio of the population and the proportion of infected females. A second, smaller sub-sample (~50-80 individuals) was then frozen at -80°C for allozyme electrophoresis at a later date. This constituted the 'random' sample for allozyme analysis. Finally, any infected individuals remaining in the sample were examined to establish the cause of infection and, if enough were available, intact individuals were also frozen at -80°C. These individuals represented the 'infected' sample for allozyme analysis.





**Figure 2.1.** Map showing the localities of the 21 populations sampled during the study period.

### **2.3.2. Allozyme electrophoresis**

Samples were analysed using the cellulose acetate electrophoresis methods described in Hebert and Beaton (1993). Allozyme phenotypes were discriminated by screening for variation at four enzyme loci known to be polymorphic in *D. pulex* from this region (P. Hebert, personal communication)—aldehyde oxidase (*AO*) (EC 1.2.3.1), glucose-6-phosphate isomerase (*GPI*) (EC 5.3.1.9), mannose-6-phosphate isomerase (*MPI*) (EC 5.3.1.8) and phosphoglucumutase (*PGM*) (EC 5.4.2.2). For each locus, distinct alleles were labelled according to their mobility, with the fastest labelled '1', the next fastest '2' and so forth. A single laboratory clone of *D. pulex* was used as a marker clone (allozyme phenotype = *AO* – '11', *GPI* – '23', *MPI* – '33', *PGM* – '22').

### 2.3.3. Analysis

Regression analysis was used to look at relationships between parasite prevalence and three different continuous measures of sexuality – Genotypic Diversity Ratio (GDR), the log-transformed probability that the observed genotypic array was in Hardy-Weinberg equilibrium (LOGHW) and the population sex-ratio.

Both GDR and LOGHW have been used to establish breeding system in previous studies on N. American *D. pulex* populations (Hebert *et al.*, 1988; Hebert *et al.*, 1989; Hebert *et al.*, 1993; Hebert & Finston, 2001). The GDR of each population was calculated by dividing the observed number of multilocus genotypes (MLGs) with predicted values generated by Monte Carlo simulation. The allozyme data from the first sampling date was used in all cases. The simulation ran for 50,000 repeats and used the observed allele frequencies at each locus to produce the mean number of MLGs expected for a freely recombining, panmictic population, of sample size,  $N$ . Obligately asexual populations are expected to have low (usually  $<0.5$ ) GDR values (Hebert *et al.*, 1993).

LOGHW was calculated by log-transforming the  $P$ -values obtained from Chi-square analyses which compared the observed genotypic arrays with those expected for populations in Hardy-Weinberg equilibrium. As above, the data from the first sampling date was used in all cases. Loci regarded as monomorphic (those for which the frequency of the most common allele was  $\geq 0.9$ ) were excluded from the analysis. If a population was polymorphic at more than one locus, the mean log  $P$ -value was determined. Log probability values of around  $-2.00$  or less are normally indicative of obligate parthenogenesis (Hebert *et al.*, 1993).

The final measure of sexuality, the sex-ratio of each population, was estimated by calculating the mean proportion of males in the population relative to the mean proportion of ehippial females. Although male frequency alone has been used as an indicator of sexuality in previous Red Queen studies (e.g. Lively & Jokela, 2002; Ben-Ami & Heller, 2005), the nature of *D. pulex*'s reproductive cycle makes it potentially unreliable here; in sexual populations, males are normally only produced during ehippia production, so lack of males may simply indicate that the cues responsible for initiating the sexual phase of the life-cycle have not yet transpired. However, males would be expected in a sexual population containing ehippial females. As such, any populations in which ehippial females were not present were excluded from the regression analyses. I found that this did not quantitatively affect the results.

To test for evidence of frequency-dependent patterns of parasitism, I sought to determine if common host genotypes tended to be significantly over-parasitized. For this, the genotypic compositions of the random and infected samples were compared using contingency table analysis. Genotypes with a frequency of 5% or less were always pooled together into a single 'rare' clonal class. If a significant difference was found between the infected and random samples, Fisher's Exact Tests were performed on individual clones to establish which were 'overparasitized' and which were 'underparasitized'. In all cases, sequential Dunn-Sidak tests were used to adjust for multiple comparisons (Sokal & Rohlf, 1995).

## 2.4. Results

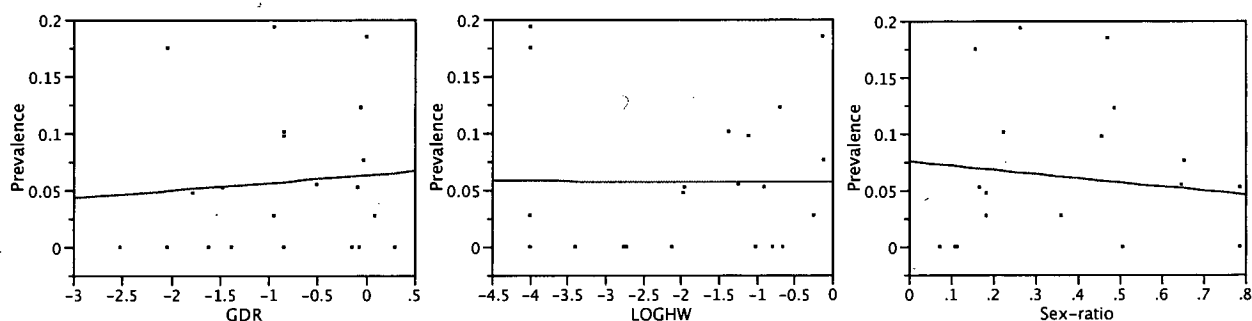
Data for each population is summarised in Table 1.1. Mean parasite prevalence ranged from 0% to 3.73%, averaging 0.73% overall. Three different parasite species were found in total (two microsporidians and one bacteria), though only one parasite species at a time was ever present in a single population. All three species infected the swimming muscles, fat cells and ovaries, though the bacteria were also found to infect the haemolymph in the later stages of infection. Attempts were made to identify each species using Green (1974), however these were unsuccessful. Due to their low prevalence and infrequent distribution, the data for all three species was pooled together for analysis. As such, parasite prevalence showed no relationship with either sex-ratio ( $r^2 = 0.021$ ,  $P = 0.543$ ), GDR ( $r^2 = 0.007$ ,  $P = 0.711$ ) or LOGHW ( $r^2 < 0.0001$ ,  $P = 0.985$ ) (Fig. 2.1). Sex-ratio showed a significant relationship with both GDR ( $r^2 = 0.6$ ,  $P < 0.0001$ ) and LOGHW ( $r^2 = 0.44$ ,  $P = 0.001$ ), and GDR and LOGHW were also highly correlated ( $r^2 = 0.599$ ,  $P < 0.0001$ ).

Table 1.1. Mean levels of parasite prevalence and measures of sexuality for 21 population of *Daphnia pulex* from south-west Ontario.

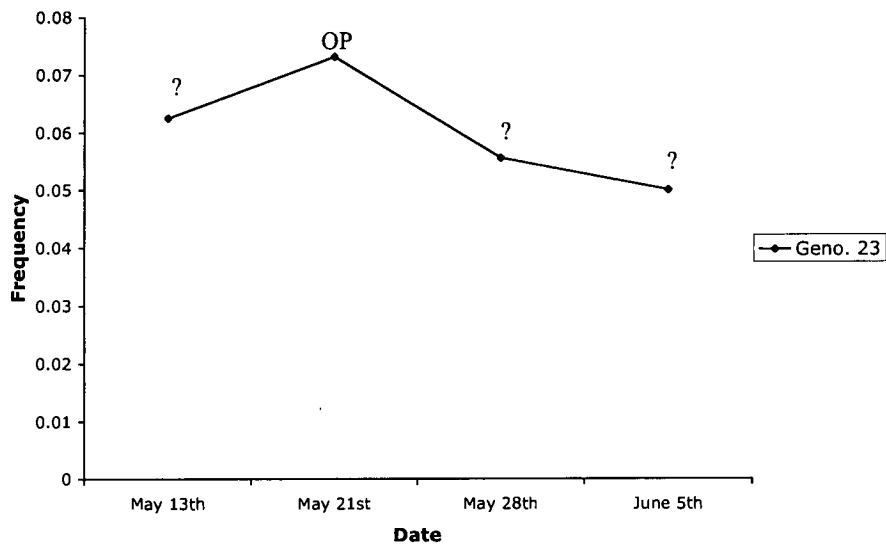
Population	Region	Observed MLG (N)	Predicted MLG	GDR	LOGHW	Sex-ratio (proportion male)	Mean Prevalence (Total N)
Sar4	Sarnia	14 (57)	16.12	0.86	-1.101	0.195	0.01 (1446)
Sar4a	Sarnia	25 (44)	21.44	1.17	-1.244	0.362	0.003 (1684)
Sar4b	Sarnia	19 (58)	18.51	1.03	-0.127	0.204	0.034 (1999)
Sar5	Sarnia	2 (40)	5.67	0.35	-4	0.033	0.001 (1067)
Sar5a	Sarnia	11 (57)	9.86	1.12	-0.116	0.37	0.006 (1573)
Sar5b	Sarnia	21 (43)	21.17	0.99	-0.652	0.235	0 (1058)
Sar5c	Sarnia	26 (48)	28.21	0.92	-0.694	0.218	0.015 (1237)
Sar6	Sarnia	12 (40)	10.45	1.15	-0.246	0.123	0.001 (1308)
Sar7	Sarnia	1 (40)	3	0.33	-4	0.068	0.037 (786)
Turkey Pt.	Long Point	3 (40)	8.09	0.37	-2.112	0.5	0 (1097)
LP7	Long Point	19 (44)	17.85	1.06	-1.016	0.5	0 (707)
LP8b	Long Point	15 (40)	17.28	0.87	-0.903	0.5	0.003 (1940)
LP9a	Long Point	21 (55)	17.33	1.21	-0.793	0.5	0 (2525)
Ron1	Rondeau	8 (60)	26.83	0.3	-2.751	-	0 (177)
Ron2	Rondeau	11 (50)	32.09	0.34	-1.974	0.034	0.002 (1301)
Ron4	Rondeau	4 (80)	18.82	0.21	-4	0.013	0 (1190)
Ron5	Rondeau	4 (80)	16.12	0.25	-4	0.024	0.031 (1935)
Ron7	Rondeau	10 (59)	44.61	0.22	-2.71	0.012	0 (1350)
SP2	Rondeau	15 (43)	29.81	0.5	-3.385	0.006	0 (1129)
Eb1	Rondeau	17 (60)	29.37	0.58	-1.375	0.049	0.01 (2017)
Eb2	Rondeau	21 (60)	33.96	0.35	-1.958	0.027	0.003 (2334)

Note: Values in parentheses in the 'Observed MLG' column indicate the number of individuals allozymed in order to estimate MLG values, GDR and LOGHW. Values in parentheses in the 'Mean Prevalence' column indicate the total number of individuals sampled for each population over the whole study period, in order to estimate sex-ratio and mean prevalence.

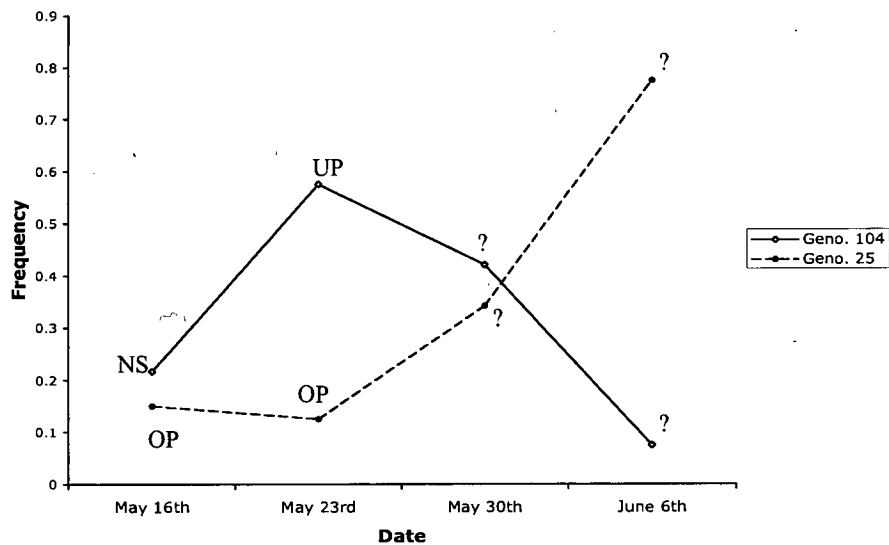
Contingency table analysis found significant differences in clonal frequencies between the infected and random samples in two of the sixteen infected populations—Sar5c and Eb2. In both populations further analysis detected the presence of both overparasitized and underparasitized clones. In population Sar5c, differences were found in the May 21<sup>st</sup> sample, with clone 23 being overparasitized (Fisher's Exact Test;  $P=0.0089$ ) (Fig. 2.3a) and the pooled 'rare' clones being underparasitized ( $P=0.0204$ ). Unfortunately this was the only date upon which a large enough infected sample could be collected from this population, so it was not possible to follow these genotype-specific infection patterns any further. In population Eb2, genotype 25 was found to be overparasitized on two consecutive sampling dates (May 16<sup>th</sup> and 23<sup>rd</sup>;  $P=0.0032$  and  $P=0.0015$ , respectively), whereas genotype 104 was found to be underparasitized on one of these sampling dates (May 23<sup>rd</sup>;  $P=0.0045$ ) (Fig. 2.3b). No significant interaction was found for genotype 104 on May 16<sup>th</sup>. Again the paucity of infected individuals in the collected sample meant that no further comparisons could be made after the second sampling date.



**Figure 2.2. Bivariate plots of mean parasite prevalence against GDR, LOGHW and sex-ratio. GDR and LOGHW data has been log-transformed, prevalence and sex-ratio data was arcsine square-root transformed.**



**Figure 2.3a.** Frequency changes of genotype 23 in population Sar5c. Overparasitism (OP) of this genotype was detected on May 21<sup>st</sup>. '?' indicates dates when not enough infected individuals were available for analysis.



**Figure 2.3b.** Frequency changes of genotypes 25 and 104 in population Eb2. Genotype 25 was found to be overparasitized (OP) on May 16<sup>th</sup> and 23<sup>rd</sup>. Genotype 104 was underparasitized (UP) on May 23<sup>rd</sup>. '?' indicates dates when not enough infected individuals were available for analysis, 'NS' indicates no significant interaction.

## 2.5. Discussion

The patterns of parasite prevalence observed in this study do not support the hypothesis that parasite-mediated selection has been responsible for the maintenance of sexual reproduction across this particular region of *D. pulex*'s range. Across 21 populations there was no significant relationship between parasite prevalence and three different measures of sexuality. Across populations, infection levels were always low (0-4%), whereas the amount of sexuality appeared to vary considerably (1-50% males).

The observed positive relationship between sex-ratio and genetic estimates of sexuality (GDR and LOGHW) suggests that all three provide reliable estimates of the amount of sexual reproduction in a population. This is consistent with previous studies showing that male production is much reduced in obligately asexual *D. pulex* populations (Hebert *et al.*, 1989; Innes & Singleton, 2000; Innes *et al.*, 2000). Genetic variation for male production has been shown to exist among clones of *D. pulex* and Innes *et al.* (2000) have suggested that this may lead to selection against male production in obligate parthenogens. Earlier work has shown that clones producing few or no males tend to dominate populations in the areas in which they are found (Hebert *et al.*, 1989) and this is thought to be due to their ability to avoid the 'cost of males' (Innes *et al.*, 2000).

In contrast to previous field studies on parasitism in *Daphnia* populations (e.g. Little & Ebert, 1999; Duncan *et al.*, 2006), the levels of parasitism reported here suggest that parasites are not a major source of selection in these populations. At present it is not possible to explain why overall levels of parasite prevalence remained so low, as nothing is currently known about the infection and transmission dynamics of the three



microparasite species identified here. As these ponds are ephemeral (usually only lasting for 4 – 5 weeks), the study period did cover the majority of the growing season and it seems unlikely that I missed a late peak in prevalence. Repeated studies over consecutive years would be very useful for confirming the selective role of parasites in these populations. If the levels of infection observed were indicative of the usual annual patterns, this might go some way towards explaining why obligate parthenogenesis has become the dominant mode of reproduction in this particular region of North America (Hebert *et al.*, 1988; Hebert *et al.*, 1989). Assuming that parasitism does play a role in the maintenance of sex, low levels of infection risk and prevalence would favour asexual reproduction (Lively, 2001), and sex would really only be stable at such low transmission levels if the fitness effects of infection were very severe (Howard & Lively, 1998). Measures of parasite prevalence could greatly underestimate the true impact of parasites because the most virulent parasites, i.e. those that kill quickly, will typically be found at low prevalence. Additionally, parasites might play a role in breeding system variation if their fitness consequences interact with other selective pressures, such as deleterious mutations (West *et al.*, 1999; Killick *et al.*, 2006). The effect these particular parasite species have on host fitness is not known, though preliminary observations would suggest that they do have some impact on host fecundity, as none of the infected females collected were carrying broods.

Two out of the twenty-one populations showed significant differences in clonal composition between infected and uninfected samples, which suggests that some degree of parasite-mediated selection may be taking place despite the low levels of infection. However, interpretation of these patterns is confounded by several factors. Firstly, for

most of the infected populations sampled the low levels of infection meant that only a single parasitised sample could be analysed. Most Red Queen models predict that patterns of frequency-dependent selection will be time-lagged, meaning that at a given time point associations between host genotypes and parasitism may be too weak to detect (Dybdahl & Lively, 1998). Therefore, it is possible that single time-point sampling will miss any associations that do arise. As such, the data presented here can only provide a conservative indication as to the frequency with which these patterns are occurring. Associations may also have been missed due to the fact that only a small number of infected individuals (usually <16) could ever be collected from the population samples. Unless the associations were particularly strong, it is unlikely that they will be detected when only a small number of infected individuals have been analysed, and this will be exacerbated by the low levels of infection. As Little and Ebert (1999) have highlighted, when parasite prevalences are low comparisons between infected and uninfected classes are likely to be uninformative without much larger sample sizes.

One further inconsistency arises from the seemingly contradictory changes in genotype frequency observed for the overparasitized clones in populations Sar5c and Eb2, and again the lack of infected samples make it difficult to account for the cause(s) of these changes. Clone 23 in population Sar5c was overparasitized when it was at its highest frequency and then went on to show a decline over the remaining study period, which is consistent with the idea that common clones will find themselves being selected against (e.g. Dybdahl & Lively, 1998). However, clone 25 in population Eb2 shows the opposite pattern; overparasitism occurs when the clone is at its lowest frequency and this is then followed by a dramatic increase in frequency over the remainder of the study

period. Although it could be argued that overparasitism is the reason this clone remained at such low frequencies over the first two weeks, it is not possible to ascertain the reason behind its sudden rise in frequency over the remaining two weeks. A variety of environmental factors are known to influence genotype-frequency changes in natural *Daphnia* populations (Hebert & Ward, 1976; Korpelainen, 1986; Carvalho & Crisp, 1987; Geedey *et al.*, 1996), so a number of factors may have been responsible. Again, due to the paucity of infected samples the patterns observed are not readily open to interpretation.

The results, as they stand, suggest that parasitism does not play a major role, as parasite prevalences were uniformly low across all populations. Whether or not such low prevalences are consistent across years remains to be seen, as parasite prevalences in other *Daphnia* species have been shown to vary widely both within and between seasons (Little & Ebert, 1999; Duncan *et al.*, 2006). The reasons for this are not always obvious, though climate has been linked to annual fluctuations in parasite prevalence in *D. magna* populations (Duncan *et al.*, 2006). If infection levels in the *D. pulex* populations studied are prone to climate-linked fluctuations then the role of the Red Queen immediately comes into doubt anyway, regardless of the potential for parasites to have more of an impact in climatically-favourable years. The threat of invasion posed by asexual clones is likely to be consistent across years, and as such any advantage provided to sexuals by parasite-mediated selection will be compromised if it is only effective under certain conditions.

If parasites are not responsible for maintaining breeding system variation in this region, then it is not immediately obvious what is. The populations surveyed all occupied

very similar habitats (temporary melt-water ponds in deciduous forest) and were around for comparable periods of time. The Great Lakes region is almost exclusively dominated by obligate parthenogens, yet cyclical populations have remained sexual despite evidence they have repeatedly been exposed to 'contagiously' asexual clones (Crease *et al.*, 1989). It has been suggested that these populations may possess a gene that suppresses the action of the meiotic-suppressor (Hebert *et al.*, 1989). However, in the absence of any obvious advantage to sexuality, it is unclear why such a gene would persist.

## **Chapter 3. Testing the pluralist approach to sex: the influence of environment on synergistic interactions between mutation load and parasitism in *Daphnia magna*.**

### **3.1. Abstract**

Both deleterious mutations and parasites have been acknowledged as potential selective forces responsible for the evolutionary maintenance of sexual reproduction. The Pluralist Approach to sex proposes that these two factors may have to interact synergistically in order to stabilise sex, and one of the simplest ways this could occur is if parasites are capable of causing synergistic epistasis between mutations in their hosts. However, a wide range of environmental factors are known to influence the effects of both deleterious mutations and parasitism, so the nature of the interaction may depend upon the organisms' environment. Using chemically-mutated *Daphnia magna* lines, I examined the effects of mutation and parasitism under a range of temperature and food regimes. I found that although parasites were capable of causing synergistic epistasis between mutations in their hosts, these effects were dependent upon an interaction between parasite genotype and temperature.

### 3.2. Introduction

One of the greatest challenges for evolutionary biology is explaining the evolutionary maintenance of sexual reproduction, and the associated process of genetic recombination (Maynard Smith, 1978; Bell, 1982; Barton & Charlesworth, 1998; West *et al.*, 1999; Burt, 2000; Otto & Lenormand, 2002). The problem is that asexual females can potentially produce twice as many daughters as sexual females, resulting in a 'two-fold' cost of sex. In addition, recombination breaks up favourable gene combinations that have passed the test of natural selection. Yet, despite these costs, sex is widespread throughout Eukaryotic taxa.

Although numerous explanations for sex have been suggested, there are currently two leading hypotheses (Kondrashov, 1993; West *et al.*, 1999). First, the Mutational Deterministic (MD) hypothesis states that sex is advantageous because it allows deleterious mutations to be eliminated more efficiently (Kondrashov, 1982; Charlesworth, 1990). The MD hypothesis requires that each additional mutation leads to a greater decrease in fitness than the last (termed synergistic epistasis), because this leads to negative linkage disequilibrium which slows down the removal of deleterious mutations- here sex is advantageous because it destroys linkage disequilibria (Kondrashov, 1982; Charlesworth, 1990). Second, the Red Queen hypothesis states that sex provides an advantage in antagonistic biotic interactions (Bell, 1982). It is usually assumed that selection by coevolving parasites against common host genotypes provides the antagonistic coevolutionary dance that is required to drive the Red Queen (Hamilton *et al.*, 1990; Peters & Lively, 1999).

However, several lines of evidence suggest that a pluralist approach may be required to explain sex, with both (or more) of these mechanisms in operation (West *et al.*, 1999). In particular: (1) Empirical estimates suggest that the deleterious mutation rate per genome per generation in a high proportion of sexual species is likely to be too low to fully explain sex (Keightley & Eyre-Walker, 2000); (2) Experimental data suggests that synergistic epistasis is not ubiquitous (Mukai, 1969; Elena & Lenski, 1997; Whitlock & Bourget, 2000; Peters & Keightley, 2000; Rivero *et al.*, 2003); (3) Theoretical analyses suggest that the Red Queen hypothesis requires extremely strong selection (Howard & Lively, 1994; Otto & Nuismer, 2004) of a form that is not commonly met in natural populations (Little, 2002). Furthermore, empirical work has also shown that genotype-specific infection patterns can be affected by environmental conditions (Blanford *et al.*, 2003; Stacey *et al.*, 2003; Mitchell *et al.*, 2005), which could interfere with Red Queen dynamics.

A pluralist approach can make sex easier to explain in at least three ways (West *et al.*, 1999). First, in a purely additive world, multiple mechanisms make it easier to fully balance the two-fold cost of sex (Howard & Lively, 1994, 1998). Acting together, each mechanism can play an important role even if it requires extreme, and possibly unreasonable, assumptions in order to fully explain sex on its own. Second, the dynamics of different hypotheses can complement each other (Lively & Howard, 1994). For example, by repeatedly driving asexual genotypes down in frequency (in effect bottlenecking them) Red Queen dynamics can provide a short-term advantage to sex by providing time for and speeding up the accumulation of deleterious mutations (Howard & Lively, 1994, 1998; see also Peck, 1994). Third, the fitness consequences of parasites

and mutations may interact synergistically (Peters, 1999; West *et al.*, 1999). The simplest way this could occur is if parasites lead to increased selection against deleterious mutations or are the factor that drives synergistic epistasis between mutations (West *et al.*, 1999; Cooper *et al.*, 2005; Buckling, 2006). However, a further possibility is that there are complex interactions between parasite infection, mutation load and environmental factors, where the shape of the relationship between mutation load and fitness varies depending upon parasite and host genotype, and the prevailing environmental conditions. Understanding these interactions is important, as the general application of the pluralist approach may actually be hindered if it is only relevant under given circumstances.

Here, I carry out the first empirical test of whether the fitness consequences of deleterious mutations and parasites interact, and vary with both parasite genotype and environmental conditions. I performed experiments on *Daphnia magna*, where there is already evidence that the fitness consequences of parasite infection depend upon an interaction between host and parasite genotype, as required by the Red Queen hypothesis (Carius *et al.*, 2001; Mitchell *et al.*, 2005). I experimentally manipulated mutation load with an artificial mutagen, as has proved a useful method in studies on other organisms (Peters & Keightley, 2000; Rivero *et al.*, 2003). Since the MDH relies upon mildly deleterious mutations in the heterozygous state (Rivero *et al.*, 2003), this also allowed me to avoid the confounding effects of inbreeding encountered in similar studies on *D. magna* (Haag *et al.*, 2003; Salathé & Ebert, 2003). My specific aim was to determine the relationship between fitness and the number of deleterious mutations, and how this varies



with: (a) parasite infection; (b) parasite genotype; (c) environmental conditions (temperature and food availability).

### **3.3. Materials and methods**

#### **3.3.1. Study system**

*Daphnia magna* is a cyclically parthenogenetic planktonic crustacean commonly found in freshwater ponds. Gender is environmentally determined, and *D. magna* will reproduce sexually given the right environmental cues. However, it is possible to maintain clonal lineages indefinitely in the laboratory by maintaining them under the appropriate conditions.

The *D. magna* clone used in this study was a long-term laboratory clone that originated from a pond near Gaarzefeld, northern Germany. The use of a single genotypic 'clone' allowed me to control the effects of host genetic background in a more precise way than is normally possible in studies on other sexual species. *Daphnia* were kept in jars containing 200ml of *Daphnia* medium (Aachener Daphnien Medium, as described by Klüttgen *et al.* (1994)) at 20°C in a 12L : 12D cycle, with six *Daphnia* per jar. The animals were fed  $5 \times 10^6$  algal cells (*Scenedesmus* sp.) per *Daphnia* per day and the medium was changed every other day. New generations were started using six second-clutch neonates from each jar.

*Pasteuria ramosa* is a horizontally-transmitted bacterial endoparasite of *D. magna*. Infection occurs when water-borne spores, released from dead hosts, are ingested by the animals as they filter-feed. Development of infection is associated with a severe

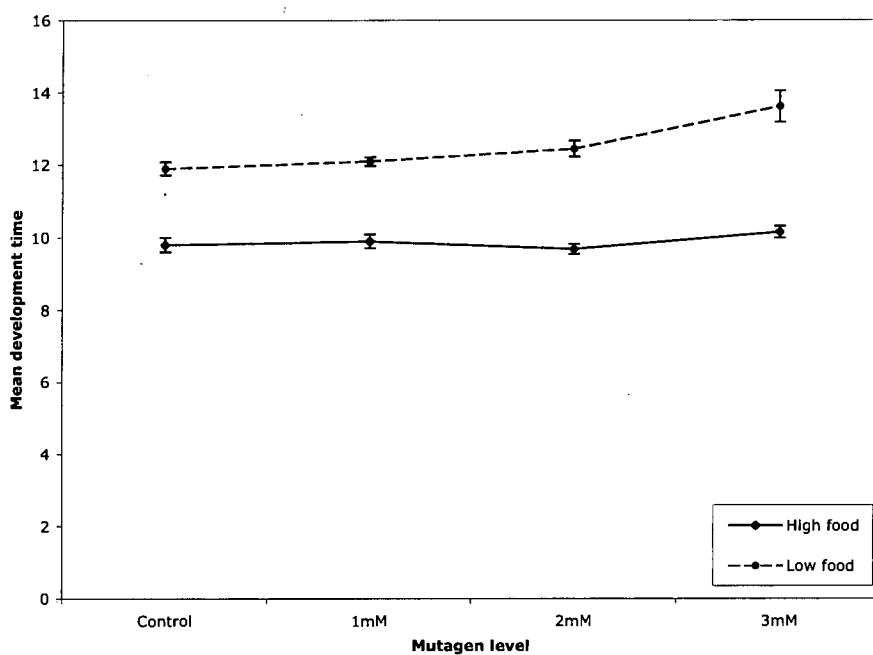
reduction in host fecundity and in many cases the host is permanently sterilised. The three *P. ramosa* isolates used in this study (designated Sp1, Sp7 and Sp13) originated from the same location as the host clone, and were isolated during a previous study examining strain-specific patterns of infectivity (Carius *et al.*, 2001). These strains are known to vary substantially in their infectivity when exposed to this particular host clone – strain Sp13 is relatively avirulent (0-2% infection), Sp1 is highly virulent (80-100% infection) and Sp7 is of intermediate virulence (20-60% infection) (Chapter 5, Fig. 5.1). A short time prior to the experiments, solutions containing 50,000 spores ml<sup>-1</sup> were made up for each parasite strain. These were frozen at -20°C until required, after which they were stored at 4°C.

### 3.3.2. Mutagen preparation and dosage assessment

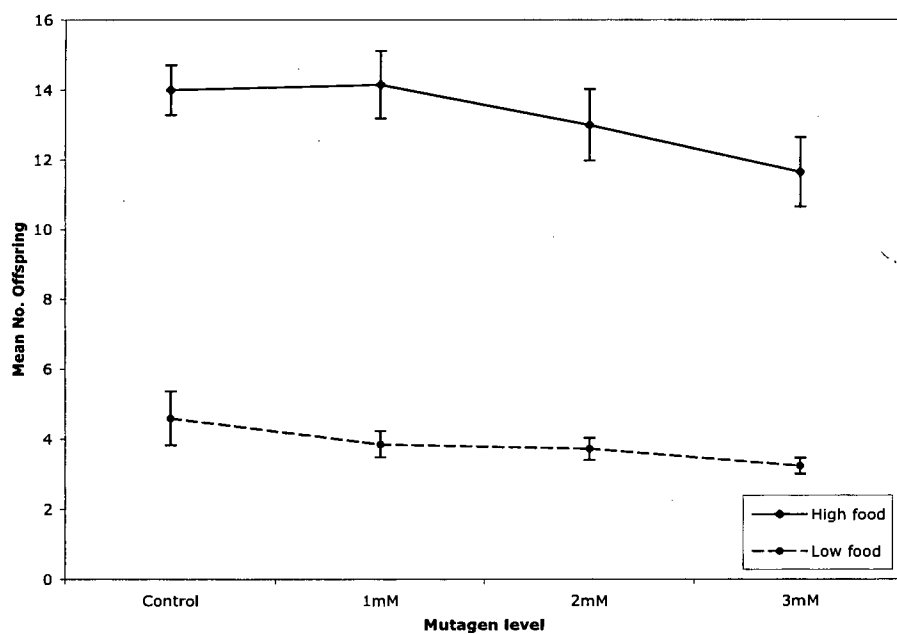
Mutations were induced using *N*-ethyl-*N*-nitrosourea (ENU), a chemical mutagen known to be highly efficient at inducing point mutations (Solnica-Krezel *et al.*, 1994; Nolan *et al.*, 2000). ENU is an alkylating agent capable of inducing a high-level of locus-specific recessive mutations (Solnica-Krezel *et al.*, 1994). In this respect, when compared to other mutagenising agents the mutations arising from ENU mutagenesis bear a greater similarity to those arising naturally in the genome. For example, UV and ionizing radiation can both be used to induce deleterious mutations over short time periods but their effects are usually substantial and tend to occur at a chromosomal, rather than at a base, level (Hartl & Jones, 1999). The exact nature of the mutations induced by ENU mutagenesis appears to depend on the organism being treated; in mice transitions and

transversions at AT sites are common (Solnica-Krezel *et al.*, 1994), whereas in *E. coli* the majority of mutations induced by ENU are GC→AT transitions (Richardson *et al.*, 1987). However, given that these are the commonest types of mutation to arise during the replication process (Hartl & Jones, 1999), ENU treatment would appear to be a valid method of rapidly inducing point mutations.

Before any of the experimental lines had been exposed to the mutagen, a pilot study was conducted in order to establish the concentration of ENU required to induce heritable and significantly deleterious mutations in female *Daphnia*. Four different mutagen treatment groups were established – Control (0mM), 1mM, 2mM and 3mM- with twenty-four females per treatment. The mutagen was prepared by injecting 85ml of *Daphnia* medium (buffered to pH <6 using 5mM MES) into a 1g ENU Isopac® (Sigma #3385), producing a ~100mM solution, which was then diluted accordingly. Fitness assays were conducted five generations post-treatment, to exclude the possibility of that any effects on fitness were simply due to persistent environmental effects (Rossiter, 1996) caused by ENU toxicity. Time to first brood production and first brood size were used as fitness measures. Fitness was found to decline with increasing mutagen concentration (Figs. 3.1 and 3.2), though further analysis of the data suggested that only the 3mM treatment had a significant effect on fitness (Table 3.1).



**Figure 3.1. Effect of mutation treatment and food conditions on mean development time.** Data is untransformed for clarity, bars represent the standard error.



**Figure 3.2. Effect of mutation treatment and food conditions on mean first brood size.** Data is untransformed for clarity, bars represent the standard error.

Contrast	SS	F	P
Control – 1mM	3.64	0.58	0.448
Control – 2mM	11.21	1.78	0.185
Control – 3mM	39.57	6.28	0.013

**Table 3.1. Results of LS means contrast analysis comparing mean offspring production in the mutated and control lines. In all cases, nom/den d.f. = 1, 134;  $n = 141$ .**

### 3.3.3. Mutagenesis

I exposed forty-eight four-day old females to the mutagen by placing them individually into tubes containing 10ml of 3mM ENU solution. Exposure lasted for 1 hour, after which the animals were moved to a small tank containing fresh *Daphnia* medium to recover and rinse off the mutagen. This ‘rinse and recover’ step was repeated three times in order to remove as much of the mutagen as possible. Individual females were then moved to 200ml jars and the lines were maintained as described above.

One generation later, twenty-four lines were selected at random and one female from each of these lines was selected to go through another round of mutagenesis. These double-treated lines were classed as ‘M2’ lines, whereas the remaining twenty-four single-treated lines constituted the ‘M1’ group. A further twenty-four untreated lines were maintained as unmutagenised controls. Importantly, this methodology means that the additional mutations were added separately in each line, making them real replicates (West *et al.*, 1998).

The experiments were carried out approximately 11 (Experiment 1) and 14 (Experiment 2) *Daphnia* generations after the mutagenesis treatments. Other experimental work conducted in the intervening period demonstrated that the effects of

the mutagenesis were heritable and had been retained during this time (S.C. Killick, unpublished data). However, the number of mutant lines was significantly reduced during the first few post-treatment generations, largely due to high levels of sterility and juvenile mortality. As a result, only fourteen M1 lines and eight M2 lines were available for further study. In order to keep the number of lines used for each mutation level fairly even, twelve of the untreated control lines were randomly selected for use in the experiments.

#### **3.3.4. Experimental procedure**

For the pre-experimental maternal generation, the required number of jars was set up for each of the 34 lines, each containing six 2<sup>nd</sup> brood females. These were maintained at 20°C, in a 12L : 12D cycle, fed  $5 \times 10^6$  algal cells *Daphnia*<sup>-1</sup> day<sup>-2</sup> and the medium was changed every other day.

Experimental replicates were established using 2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup> brood offspring, depending on the number of neonates (<24 hrs old) produced per brood. As such, the different parasite spore treatments were always applied between offspring from the same maternal line but a split-brood design was not possible due to insufficient clutch sizes.

For each replicate, the required number of neonates was initially placed into a 60ml jar containing a small quantity of sand, 50ml of *Daphnia* medium and 1ml of the appropriate spore solution (1ml of *Daphnia* medium was added to the controls). This constituted the infection period. Each jar was randomly allocated a position within a tray

and placed into an incubator. Jars were rotated within trays and trays were rotated among shelves daily to minimize positional effects.

Following the infection period, the animals were moved to larger jars containing 200ml of *Daphnia* medium. This constituted the post-infection period. Again, to remove positional effects, jars were randomised within trays and both jars and trays were rotated daily. Medium was changed every other day, and the number of offspring present and number of adult females remaining in each jar was recorded. At the end of each experiment, the mean total number of offspring produced per female (i.e. the total number of offspring produced over the study period divided by the number of females in the jar) was calculated for each replicate.

### **3.3.5. Experiment 1 – mutation, parasitism and food level**

For each of the 34 lines, two maternal lines were established and assigned to either the ‘Low’ food or ‘High’ food group. Offspring from these lines were then randomly assigned to one of four different parasite spore treatments: Control (no spores), Sp1, Sp7 or Sp13. Each replicate was started with six neonates and 1ml of the appropriate spore solution ( $2.5 \times 10^4$  spores  $\text{ml}^{-1}$ ) was added to each jar. The animals in both treatments were fed  $5 \times 10^5$  algal cells *Daphnia*<sup>-1</sup> day<sup>-2</sup> during the infection period, which lasted for seven days. During the post-infection period, replicates in the ‘High’ and ‘Low’ food groups were fed  $3 \times 10^6$  algal cells *Daphnia*<sup>-1</sup> day<sup>-2</sup> and  $1 \times 10^6$  algal cells *Daphnia*<sup>-1</sup> day<sup>-2</sup>, respectively. The animals were maintained at 20°C in a 12L : 12D cycle throughout, and each replicate was run for a total of 28 days.

### **3.3.6. Experiment 2 - mutation, parasitism and temperature**

For each of the 34 lines, three maternal lines were established and each was assigned to one of three temperature treatments (15°C, 20°C, 25°C). Offspring from these lines were then randomly assigned to one of four different parasite spore treatments: Control (no spores), Sp1, Sp7, and Sp13. Each replicate was started with eight neonates and 1ml of the appropriate spore solution ( $5 \times 10^4$  spores ml<sup>-1</sup>) was added to each jar. Following Mitchell *et al.* (2005), the length of the infection period and the amount of food provided was adjusted to compensate for temperature-associated differences in metabolism and feeding rate. As such, the infection period lasted 9 days at 15°C, 7 days at 20°C and 5 days at 25°C. During this time, the animals were fed  $5 \times 10^5$  algal cells *Daphnia*<sup>-1</sup> day<sup>-2</sup> at 15°C and 20°C, and  $7.5 \times 10^5$  algal cells *Daphnia*<sup>-1</sup> day<sup>-2</sup> at 25°C. For the post-infection period, food levels were also adjusted to accommodate temperature differences:  $3 \times 10^6$  algal cells *Daphnia*<sup>-1</sup> day<sup>-2</sup> at 15°C,  $3.5 \times 10^6$  cells *Daphnia*<sup>-1</sup> day<sup>-2</sup> at 20°C and  $4.5 \times 10^6$  cells *Daphnia*<sup>-1</sup> day<sup>-2</sup> at 25°C. The length of the experiment was calculated using the 'degree-day' method described by Mitchell *et al.* (2005). Each replicate was run for a total of 500 degree-days: 33 days at 15°C, 25 days at 20°C and 20 days at 25°C.



### **3.3.7. Analysis**

#### **3.3.7.1. Test for block effects**

Due to the fact that the animals were treated in two distinct 'blocks' (Block 1 = M1 lines & M2 lines first treatment, Block 2 = M2 lines second treatment) it was necessary to address the possibility that differences between the M1 and M2 lines could be affected by experimental error during the two applications. In theory, if the M2 lines were treated with the same concentration of ENU during the second round of exposure, and for the same period of time, the number of mutations induced in these lines would be expected to be twice that of the M1 lines. The number of mutations present in the M1 and M2 lines was estimated using data from the parasite-free (Control) lines in Experiment 2 (data from all three temperature treatments was pooled together to increase the power of the results), using a variation on the method described by Castle (1921):

$$N = 2D^2 / (Var_2 - Var_1)$$

Where  $N$  is the number of mutations,  $D$  is the difference between the means of the two treatments and  $Var$  is the variance within that treatment (D. Halligan, Pers. Comm.).

#### **3.3.7.2. Tests for epistasis**

Data from the two experiments was analysed separately due to differences in experimental treatments. My focal response variable was offspring production because

this is the main fitness consequence of *P. ramosa* infection (Ebert *et al.*, 1996). All data was ln-transformed for analysis, as this is needed to test for epistatic interactions between mutations (Charlesworth, 1990; West *et al.*, 1998).

First, I looked at the factor effects and their interactions by running generalised linear mixed models (GLMMs) fitted using the Expected Mean Squares (EMS) method. For Experiment 1, mean total offspring production was analysed using food level, mutation level and parasite treatment as fixed effects, with mutant line entered as a random effect and nested within mutagen level. For Experiment 2, mean total offspring production and mortality per replicate were both analysed using parasite treatment and mutagen level as fixed effects, and temperature as a continuous variable. Line was again entered as a random effect and nested within mutagen level. Parasite treatment was entered as a fixed effect as the differences in infectivity and virulence caused by these parasite isolates (which represent different *P. ramosa* genotypes) is well established in our laboratory (Carius *et al.*, 2001; Little *et al.*, 2006), and one of my main aims was examine potential parasite genotype-by-environment interactions, and how these in turn may affect the outcome of parasitism on mutation load. For both experiments I also analysed the effect of parasite treatment with the control (parasite-free) lines removed, in order to see if the effects of the different strains varied.

If deleterious mutations are acting independently, fitness is expected to decline in a linear fashion when plotted on a log scale. However, under synergistic epistasis, in which each additional mutation leads to a greater reduction in fitness than the last, the fitness function is expected to be non-linear, with the slope declining more steeply as the number of mutations increases (Charlesworth, 1990). To test for non-linearity, I

compared the least square means for each mutation level using the contrast methods described in Underwood (1997). If fitness was declining in a linear fashion, then the difference between the LS means of the M1 and M2 lines should not be significantly different from the difference between the Control and M1 lines, i.e.  $(C - M1) = (M1 - M2)$ . However, if mutations were interacting synergistically the M1 - M2 difference would be expected to be significantly higher than the Control - M1 difference, i.e.  $(C - M1) < (M1 - M2)$ .

If I found evidence for epistasis, I analysed the data further using standard regression analysis in order to verify non-linearity and examine the effects of the individual parasite strains. The linear and quadratic terms were fitted sequentially to the data. If the quadratic term ( $\ln Y = c + \alpha m + \beta m^2$ ; where  $Y$  is fitness and  $m$  is the number of mutation episodes) was significant (when the linear term was also included in the model), the fitness function was deemed to be non-linear and the interaction between mutation levels was considered to be epistatic (Elena & Lenski, 1997; West *et al.*, 1998; Peters & Keightley, 2000; Rivero *et al.*, 2003; Fry, 2004). Under these circumstances mutagen level had to be entered as a continuous variable, meaning the random effects of line could not be nested within mutagen level. However, I found that my results were still in agreement with the contrast analysis.

## 3.4. Results

### 3.4.1. Test for block effects

The M1 lines were estimated to be carrying 1.39 mutations on average, whereas the M2 lines were estimated to be carrying an average of 2.88 mutations. Given that the value for the M2 lines is very close to double that of the M1 lines ( $2.88/1.39 = 2.07$ ) it seems safe to assume that the dose of ENU received during the second block of treatments was similar to that received during the initial block of mutagenesis. As such, block effects do not appear to have any influence over patterns observed in the data.

### 3.4.2. Experiment 1 – mutation, parasitism and food level

There were highly significant effects of mutation load ( $F_{2,39} = 54.74, P < 0.0001, N = 188$ ), parasite treatment ( $F_{3,39} = 129.67, P < 0.0001$ ) and food availability ( $F_{1,47.27} = 244.83, P < 0.0001$ ) on offspring production (Table 3.2; Fig. 3.3). With the Control (parasite-free) lines removed from the analysis the effect of parasite treatment remained significant ( $F_{2,22} = 58.43, P < 0.0001$ ), confirming that levels of virulence differed between strains. However, parasite treatment showed no significant interaction with either food treatment ( $F_{3,39} = 0.95, P = 0.427$ ) or mutation load ( $F_{6,74.14} = 1.37, P = 0.250$ ). A similar result was obtained with the Control lines removed (Parasite x Food:  $F_{2,22} = 0.03, P = 0.975$ ; Parasite x Mutation:  $F_{4,40.77} = 1.22, P = 0.331$ ). Food treatment showed a significant interaction with mutagen level ( $F_{2,43.88} = 13.08, P < 0.0001$ ). This

appears to be due to mutational load having less of an impact under low food conditions; as can be seen in Fig. 2, the decline in fitness with increasing mutation load is much more pronounced when food is plentiful (Linear regression [all parasite treatments pooled] - High food:  $P < 0.0001$ , slope = -0.56; Low food:  $P = 0.019$ , slope = -0.19).

The contrast analysis found no significant difference between the LS mean differences ( $C - M1 = 0.4048 \pm 0.102$ ,  $M1 - M2 = 0.3322 \pm 0.122$ ;  $F = 0.737$ , N.S.), suggesting that mutations were not acting epistatically and fitness declined in a linear fashion.

Source	Df <sub>Num</sub>	Df <sub>Den</sub>	SS	MS	F	P
<i>Mutagen</i>	2	39	14.11	7.06	54.74	0.0002
<i>Parasite</i>	3	39	50.15	16.72	129.67	<0.0001
<i>Food</i>	1	47.27	31.56	31.56	244.83	<0.0001
<i>Mutagen x parasite</i>	6	74.14	1.06	0.18	1.37	0.250
<i>Mutagen x food</i>	2	43.88	3.37	1.69	13.08	<0.0001
<i>Parasite x food</i>	3	39	0.37	0.12	0.95	0.427
<i>Mut x para x food</i>	6	39	0.81	0.14	1.05	0.408
<i>Line[Mutagen]</i>	26	28.56	17.49	0.67	5.22	<0.0001
<i>Line[Mut] x para</i>	73	39	17.87	0.24	1.9	0.015
<i>Line[Mut] x food</i>	26	39	3.22	0.12	0.96	0.533

**Table 3.1. Generalised linear mixed model examining the effects of mutation load, parasite infection and food treatment. Mutagen level, parasite treatment and food level are fixed effects, with line as a random effect nested within mutagen.**

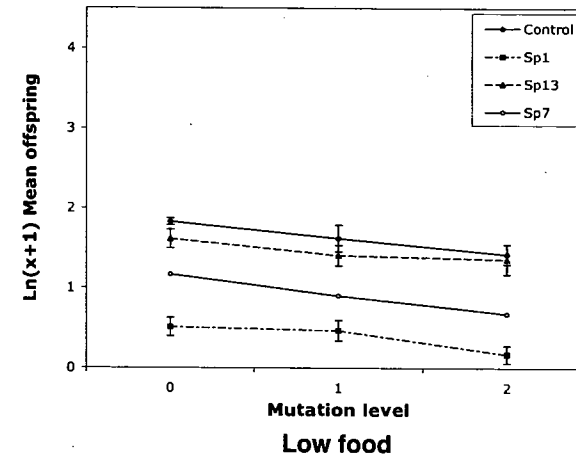
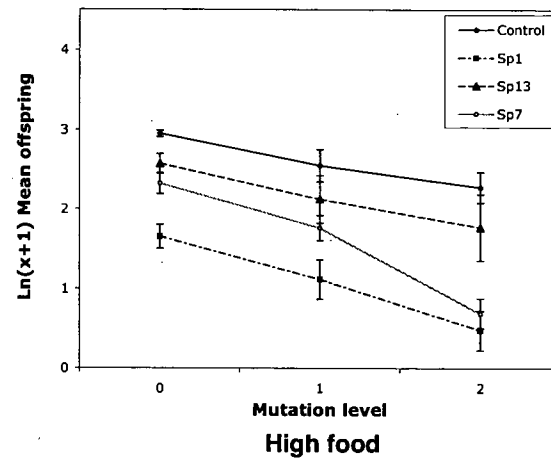


Figure 3.3.  $\ln(x+1)$ -transformed mean number of offspring produced by females from each of the three mutation treatments under different feeding regimes. Bars represent the standard errors.

### **3.4.3. Experiment 2 – mutation, parasitism and temperature**

In this experiment there were highly significant effects of mutation load ( $F_{2, 86.35} = 54.78$ ,  $P < 0.0001$ ,  $N = 365$ ), parasite treatment ( $F_{3, 227.77} = 54.38$ ,  $P < 0.0001$ ) and temperature ( $F_{1, 192} = 12.39$ ,  $P = 0.0005$ ) (Table 3.3; Fig. 3.4). Again, with the Control (parasite-free) lines removed the effect of parasite treatment remained significant ( $F_{2, 193.63} = 44.43$ ,  $P < 0.0001$ ), indicating that virulence varied between strains. Parasite treatment showed a significant interaction with both temperature ( $F_{3, 192} = 9.65$ ,  $P < 0.0001$ ), and mutation level ( $F_{6, 171.15} = 3.16$ ,  $P = 0.006$ ). With the Control lines removed the parasite treatment-by-temperature interaction remained significant ( $F_{2, 135} = 11.73$ ,  $P < 0.0001$ ) but the interaction between parasite treatment and mutation did not ( $F_{4, 159.47} = 1.1$ ,  $P = 0.360$ ). The significance of the interaction between parasite treatment and temperature in this case indicates that the effects of the individual strains varied with temperature. Mutation load was also found to significantly interact with temperature ( $F_{2, 47.26} = 18.17$ ,  $P < 0.0001$ ); the mutation load-associated decline in fitness observed in Experiment 1 was also apparent here, though it's severity varied depending on the temperature at which the lines were maintained.

I investigated the interactions (including the significant three-way interaction between mutation, parasitism and temperature) further using two-way ANOVAs for each of the three temperature treatments, with mutation load and parasite treatment as main effects. Mutation load was found to impair offspring production at 15°C ( $F_{2, 107} = 26.32$ ,  $P < 0.0001$ ) and 20°C ( $F_{2, 118} = 12.93$ ,  $P < 0.0001$ ) but not at 25°C ( $F_{2, 104} = 2.80$ ,  $P = 0.066$ ). By contrast, parasite treatment impaired offspring production at 25°C ( $F_{3, 104} =$

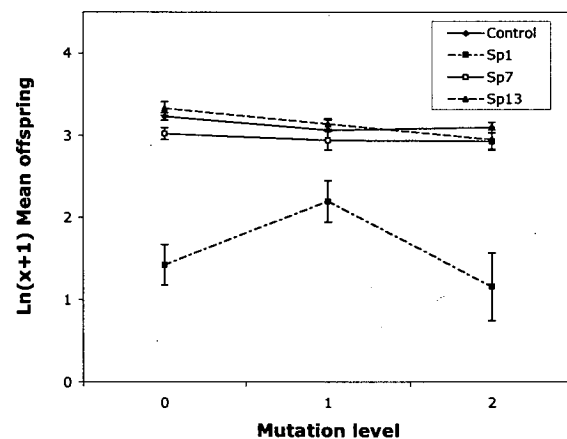


56.84,  $P < 0.0001$ ) and 20°C ( $F_{3, 118} = 16.55$ ,  $P < 0.0001$ ), but not at 15°C ( $F_{3, 107} = 2.59$ ,  $P = 0.057$ ), though this was only barely non-significant. A significant interaction between mutation level and parasite treatment was found at 25°C ( $F_{6, 104} = 2.86$ ,  $P = 0.013$ ).

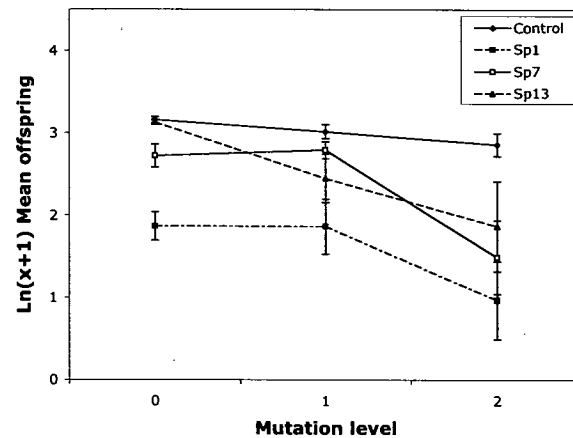
In contrast to Experiment 1, the contrast analysis found a significant difference between the LS mean differences ( $C - M1 = 0.2768 \pm 0.126$ ,  $M1 - M2 = 0.7054 \pm 0.141$ ;  $F = 16.93$ ,  $P < 0.001$ ) indicating that mutations were interacting synergistically and fitness was declining in a non-linear fashion. The results of the regression analyses are shown in Table 3.4. In agreement with the findings of the contrast analysis, the quadratic term was significant when applied to the whole data set, indicating a non-linear fitness decline overall. Separating the data into ‘parasitised’ (all three strains pooled) and ‘unparasitised’ (control) classes found that negative epistasis was only observed under conditions of parasitism. This seems to be largely due to the influence of strain Sp7, which caused epistasis in both the 20°C and 15°C treatments and also had an epistatic effect overall.

Source	Df <sub>Num</sub>	Df <sub>Den</sub>	SS	MS	F	P
<i>Mutagen</i>	2	86.35	46.53	23.27	54.78	<0.0001
<i>Parasite</i>	3	227.77	69.28	23.09	54.38	<0.0001
<i>Temperature</i>	1	192	5.26	5.26	12.39	0.0005
<i>Mutagen x parasite</i>	6	171.15	8.06	1.34	3.16	0.006
<i>Mutagen x temp</i>	2	47.26	15.43	7.72	18.17	<0.0001
<i>Parasite x temp</i>	3	192	12.29	4.10	9.65	<0.0001
<i>Mut x para x temp</i>	6	192	7.58	1.26	2.97	0.008
<i>Line[Mutagen]</i>	30	107.62	59.39	1.98	4.66	<0.0001
<i>Line[Mut] x para</i>	89	192	41.38	0.46	1.09	0.301
<i>Line[Mut] x temp</i>	30	192	18.98	0.63	1.49	0.058

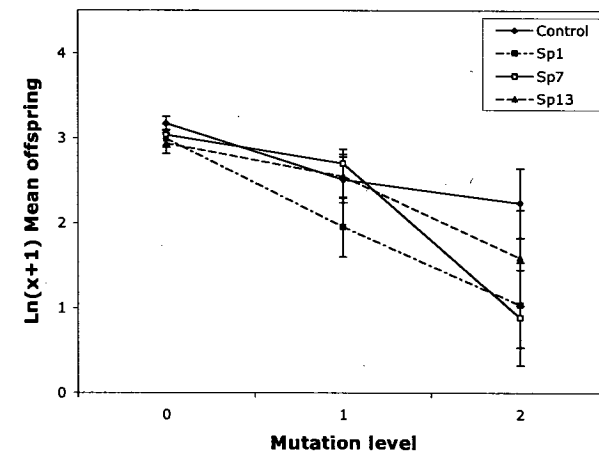
**Table 3.3.** Generalised linear mixed model examining the effects of mutation load, parasite infection and temperature. Mutagen level and parasite treatment are fixed effects, with line as a random effect nested within mutagen, and temperature as a continuous effect.



25°C



20°C



15°C

Figure 3.4. Ln(x+1)-transformed mean number of offspring produced by females from each of the three mutation treatments maintained at different temperatures. Bars represent the standard errors.

<i>Treatment</i>	Linear model		Quadratic model	
	<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>
All data	-6.07	<0.0001	-2.16	0.031
Parasite treatment				
Unparasitised (Control)	-3.20	0.002	0.78	0.436
Parasitised (pooled)	-5.64	<0.0001	-2.59	0.0102
Parasite strain				
Sp1	-2.85	0.006	-1.89	0.063
Sp7	-3.98	0.0001	-2.87	0.005
Sp13	-3.97	0.0001	-0.36	0.722
<u>25°C</u>				
Sp7	-0.72	0.48	0.28	0.779
<u>20°C</u>				
Sp7	-2.61	0.014	-2.57	0.015
<u>15°C</u>				
Sp7	-4.00	0.0005	-2.47	0.021

Table 3.4. Probability and *t*-values obtained from fitting the linear and quadratic models to  $\ln(x+1)$ -transformed fitness measurements obtained from Experiment 2. Significance of the quadratic term would suggest epistatic interactions between mutations. Significant terms are highlighted in bold for clarity.

### 3.5. Discussion

I found that the fitness consequences of deleterious mutations: (1) showed no consistent trend towards synergistic epistasis; (2) depend upon environmental conditions (food availability and temperature) and parasite infection. In Experiment 1 I found no evidence for synergistic epistasis. In Experiment 2 I found evidence for synergistic epistasis, and its extent was increased by parasite infection. The fitness consequences of parasite infection varied with genotype, and parasitism overall showed a significant interaction with mutation load. Considering environmental conditions, I found that the fitness consequences of deleterious mutations were greater when food was more plentiful (Experiment 1) and at lower temperatures (Experiment 2).

Although there was no consequence of food treatment on the effects of parasite infection, I did find a highly significant interaction between food treatment and mutation load. In both treatments fitness was found to decline in a linear manner, but with the effect of increasing mutation load more pronounced in the high food treatment. This stands in contrast to previous suggestions that the effects of mutation load should be exacerbated when individuals have to compete for resources (Kondrashov & Houle, 1994; Peck & Waxman, 2000; You & Yin, 2002; Rivero *et al.*, 2003). A possible explanation for this is that although food levels were classed as 'low', they may not have been low enough to lead to severe competition, although reducing food levels did clearly reduce fitness. Alternatively, it could be due to the way in which mutations were affecting the animals physiologically. If, for example, mutation load affected rates of nutrient uptake by reducing filter-feeding rates, then it is possible that the mutation-free

lines would be less noticeably affected by changes in food concentration. Food uptake in *Daphnia* is known to follow a Type II functional response (i.e. nutrient intake increases with increasing food concentration, but levels off due to processing constraints) (De Mott, 1982; LaMontagne & McCauley, 2001), and mutations may act to reduce the concentration at which ingestion rate asymptotes (i.e. limit food intake). Under such circumstances, the control (mutation-free) lines would be most likely to benefit from high food levels and the disparity between mutation levels would be more noticeable.

Mutation load also showed a significant interaction with temperature. Previous studies have shown that exposure to stressful temperatures can exacerbate the effects of deleterious mutations (Barnes *et al.*, 1989; Korona, 1999; Vassilieva *et al.*, 2000; Szafraniec *et al.*, 2001; but see Kishony & Leibler, 2003), though in this case none of the three temperature treatments can really be regarded as stressful; the animals are usually maintained at 20°C and the remaining two temperatures are within the temperature range normally experienced by *Daphnia* under both natural and laboratory conditions (Mitchell & Lampert, 2000; Mitchell *et al.*, 2005). As such, I actually found that mutation load reduced fitness at 15°C and 20°C, but had no significant effect at 25°C. Given that this is close to the optimal temperature for *D. magna* development (Mitchell & Lampert, 2000; Giebelhausen & Lampert, 2001), it is possible that the effects of mutation load were rendered benign under these optimal conditions, i.e. they displayed ‘conditionally neutral’ effects (Kondrashov & Houle, 1994; Elena & de Visser, 2000).

The effects of temperature on host-parasite interactions have been described in a range of other species (reviewed in Thomas & Blanford (2003)). Of the most relevance is a recent study by Mitchell *et al.* (2005), which found strong evidence of temperature-

related genotype-by-environment (G x E) interactions in the *Daphnia magna* – *Pasteuria ramosa* system. Mitchell *et al.* (2005) exposed a range of *D. magna* clones to a ‘mixed-bag’ of *P. ramosa* spores (that is, the spore solutions used were likely to contain a mixture of different parasite genotypes) under different temperature regimes, and found that not only did parasite virulence vary across the temperature range (highly virulent at high temperatures, less so at lower temperatures) but also that the susceptibility of the different clones changed between temperatures. In line with Mitchell *et al.* (2005), I found that, overall, parasitism had a high impact on host fitness at 25°C and 20°C, but less of an impact at 15°C. In the earlier study, however, it was not possible to test for parasite genotype-by-environment interactions, thus the strong parasite G x E interactions found in the present study are of particular interest. For example, all parasite strains affected host reproduction at 20°C, but at 25°C both Sp7 and Sp13 had a limited effect while Sp1 had a large effect. This strain-specific effect at 25°C indicates that temperature-dependent virulence is not likely to be due to an up-regulation of the host immune response under optimal conditions; rather, it would seem that these two isolates were simply not adapted to the higher temperature.

The main aim of this study, however, was to test the hypothesis that infection by parasites increases the extent of synergistic epistasis between mutations in their hosts. In this respect, I obtained a negative (Experiment 1) and a positive (Experiment 2) result. One of the parasite isolates (Sp7) was found to have negatively epistatic effects in Experiment 2, though these effects were influenced by temperature; Sp7 had little effect at 25°C but caused epistasis at 15°C and 20°C. This finding is of particular significance as it highlights the importance of examining pluralistic hypotheses under a diverse range

of conditions. Previous empirical tests of the Pluralist Approach to sex have failed to find evidence that parasites are capable of causing or increasing the extent of synergistic epistasis between mutations in their hosts, but these studies have utilised a restricted range of environmental conditions and did not differentiate between parasite genotypes (Peters, 1999; Salathé & Ebert, 2003; Haag *et al.*, 2003; Cooper *et al.*, 2005).

To conclude, theoretical work is required to determine the implications of our results for the evolutionary maintenance of sex and recombination. I have shown that parasite infection can lead to an increased extent of synergistic epistasis between deleterious mutations, but only under some environmental conditions. The implications of this are not clear because such interactions have not been specifically modelled, and biological details can have strong effects on the form of selection for and against sex (Otto & Nuismer, 2004). In particular, while some studies have suggested that variation in epistasis reduces the advantage of sex (Otto & Feldman, 1997; Burt, 2000), others have suggested the reverse (Agrawal, 2001; Siller, 2001)



## **Chapter 4. Specificity of the invertebrate immune response and the basis of resistance to bacterial infection in *Daphnia magna*.**

### **4.1. Abstract**

Despite increasing evidence that the invertebrate immune system is capable of the specific adaptive immune responses recognised in most vertebrates, the mechanisms underlying this specificity are still unclear. Here I investigate the nature of resistance to bacterial infection in *Daphnia magna*. The *Daphnia magna* – *Pasteuria ramosa* system shows significant host genotype-by-parasite genotype interactions, and infection studies on *D. magna* mothers and their offspring have found evidence of heritable strain-specific immunity. However, little is known about the mechanistic basis of resistance in this system, and within-generation effects have yet to be investigated. Here I examined the specificity of the *D. magna* immune response by successively exposing host lines to different strains of *P. ramosa*. I found that prior exposure to avirulent strains, before subsequent exposure to more virulent strains, could either have a slightly negative effect or slightly positive effect, depending on the initial strain encountered. I also found no evidence of resistance-associated fitness costs.

## 4.2. Introduction

The importance of an effective immune response can never be overestimated. Parasites are ubiquitous in nature, and as such it is in the best interests of most organisms to have some means of defending themselves. The field of immunology is heavily focused on the vertebrate immune response, but of course invertebrates also have a variety of humoral and cellular responses at their disposal. These range from the cellular encapsulation reaction commonly used against larger multicellular parasites (Carton & Nappi, 1997) to the release of antimicrobial peptides (AMPs) in response to infection by microorganisms (Bulet *et al.*, 1999). Until relatively recently, the invertebrate immune system was not believed to be capable of the specific, memory-based immunity displayed by the vertebrate immune system (Klein, 1997; Rinkevich, 1999; Arala-Chaves & Sequeira, 2000; Smith *et al.*, 2003; Little *et al.*, 2005; Schmid-Hempel, 2005), and this was largely due to the fact that invertebrates appear to lack components homologous to the B cells, T cells and MHC molecules characteristic of vertebrate adaptive immunity. However, as Little *et al.* (2005) have argued, this does not mean that invertebrates lack immunological memory and specificity, they may simply have evolved mechanisms similar in function but different in structure. Indeed, there is increasing phenomenological evidence that the invertebrate immune response is capable of a high degree of specificity and even memory.

For instance, genetic variation for resistance and virulence has been found in a variety of invertebrate host-parasite systems (Henter & Via, 1995; Orr & Irving, 1997; Webster & Woolhouse, 1998; Ebert *et al.*, 1998; Kraaijeveld & Godfray, 1999; Schmid-

Hempel & Funk, 2004; Lambrechts *et al.*, 2005; Schulenburg & Ewbank, 2005), and in some of these systems there is evidence that this variation relies on specific interactions between host and parasite genotypes (Carius *et al.*, 2001; Schmid-Hempel & Funk, 2004; Lambrechts *et al.*, 2005; Schulenburg & Ewbank, 2005). Such interactions are unlikely to arise in the absence of an immune response capable of distinguishing between different parasite genotypes, as this level of discrimination is only likely to occur in an immune system possessing a significant level of specificity.

Infection studies on a range of invertebrate taxa have also shown that hosts may be better protected against pathogen infection if they have previously experienced an immune challenge. The specificity of this 'immune priming' has been found to vary considerably. At a very general level, it has been shown that prior exposure to various immunogenic compounds (e.g. lipopolysaccharides, glucans, peptidoglycans, bacterins) can increase general resistance to microbial infection (Smith *et al.*, 2003; Moret & Siva-Jothy, 2003) or enhance later immune responsiveness. Moret & Siva-Jothy (2003), for example, found that mealworm beetles injected with bacterial lipopolysaccharides were subsequently more resistant to infection by the fungus *Metarhizium anisopliae*.

However, this sort of induced immune response is in no way specific, and may simply result from the persistence of AMPs in the haemolymph following the initial immune challenge (Schmid-Hempel, 2005). Specific immune priming has been demonstrated in copepods (Kurtz & Franz, 2003), shrimp (Witteveldt *et al.*, 2004) and waterfleas (Little *et al.*, 2003). In these cases hosts were exposed either to parasites or antigens they had previously experienced (homologous challenge) or to parasites or antigens they had no prior experience with (heterologous challenge). Hosts experiencing a homologous

challenge were found to be better protected than those experiencing heterologous challenges. The study on *Daphnia* by Little *et al.* (2003) is of particular interest, as the effects observed were transgenerational; offspring were found to be more resistant to the bacterial parasite *Pasteuria ramosa* if the strain they were exposed to was homologous to the one encountered by their mother. Although transgenerational priming of the immune system has been observed before in bumble-bees (Moret & Schmid-Hempel, 2001), the response observed was largely non-specific. In contrast, the *Daphnia* study provides evidence that invertebrates may be capable of producing effector molecules which act against specific parasite genotypes, and that these can be passed on in a similar manner to the way in which vertebrates pass antibodies on to their offspring (Hanson, 1999; Rollier *et al.*, 2000; Little *et al.*, 2003).

These findings have serious implications for the Red Queen hypothesis. According to the Red Queen, frequency-dependent selection against common host genotypes will prevent asexual clones capitalising on their two-fold reproductive advantage and out-competing their sexual counterparts (Hamilton *et al.*, 1990). This relies on the assumption that parasites will be selected to infect the commonest host genotypes, and in theory it should lead to strong selection against rapidly expanding asexual clones. However, if hosts are capable of mounting a strain-specific immune response against coevolving parasites, with previous experience immunising them against subsequent exposures, the potential for parasites to select for recombination may be substantially reduced (Little & Kraaijeveld, 2004). In particular, if asexual clones are able to confer this strain-specific immunity to their offspring, then the potential for sex to provide an advantage under circumstances of parent-offspring contagion (where offspring

are more susceptible to parasites adapted to their parental genotype (Rice, 1983)) may be rendered relatively impotent, if not irrelevant.

Here I investigate the specificity underlying patterns of resistance and virulence in the *Daphnia magna* – *Pasteuria ramosa* system. This system shows significant host genotype-by-parasite genotype interactions (Carius *et al.*, 2001), and the aforementioned study by Little *et al.* (2003) has demonstrated that *D. magna* is capable of mounting a specific immune reaction against different *P. ramosa* strains. However, within-generation effects have yet to be examined. In particular, it is not if clear the immune response of a host towards one strain of *P. ramosa* may affect subsequent exposure to other strains. In this regard, the nature of the interaction will be determined by the mechanisms underlying specificity. If specificity occurs at the recognition stage, with certain strains binding to a host receptor and initiating an immune reaction, then the infectivity of subsequent strains entering the host may be reduced; even if recognition is highly specific, the resulting immune response may still be general enough to hinder infection by other strains (Schmid-Hempel, 2005). In this case, highly infective strains would be those which manage to avoid triggering the host immune response, whereas non-infective (or those with low infection ability) would be those which are readily recognised. Alternatively, specificity may occur at the effector stage, with the host releasing molecules that are only effective against particular strains. Under such circumstances, prior exposure to one parasite strain will be unlikely to have an immunising effect against other strains.

By consecutively exposing lines from a single *D. magna* clone to *P. ramosa* strains differing in their infectivity, I aimed to establish: (a) whether the immune reaction

against one *P. ramosa* strain can affect the infection ability of a second strain; (b) the costs involved in the mounting of an active immune response.

### **4.3. Materials and methods**

#### **4.3.1. Study organisms**

*Daphnia magna* is a cyclically parthenogenetic planktonic crustacean commonly found in freshwater ponds. The *D. magna* clone used in this study is a long-term laboratory clone that originated from a pond near Gaarzefeld, northern Germany. *Daphnia* were kept in jars containing 1.5l of *Daphnia* medium (Aachener Daphnien Medium, as described by Klüttgen *et al.* (1994)) at 20°C in a 12L : 12D cycle, with 100 *Daphnia* per jar. The animals were fed  $5 \times 10^6$  algal cells (*Scenedesmus* sp.) per *Daphnia* per day and the medium was changed every other day.

*Pasteuria ramosa* is a horizontally-transmitted bacterial endoparasite of *D. magna*. Infection occurs when water-borne spores, released from dead hosts, are ingested by the animals as they filter-feed. Development of infection is associated with a severe reduction in host fecundity and in many cases the host is permanently sterilised. The four *P. ramosa* isolates used in this study (designated Sp1, Sp7, Sp8 and Sp13) originated from the same location as the host clone, and were isolated during a previous study examining strain-specific patterns of infectivity (Carius *et al.*, 2001). These strains are known to vary substantially in their infectivity when exposed to this particular host clone, and we have studied them extensively since their original description. Specifically,

strains Sp8 and Sp13 are essentially incapable of establishing infection (0-2% infection), Sp1 is highly infective (80-100% infection) and Sp7 is of intermediate infectivity (20-60% infection) (Fig. 4.1). A short time prior to the experiments, solutions containing 50,000 spores ml<sup>-1</sup> were made up for each parasite strain. These were frozen at -20°C until required.

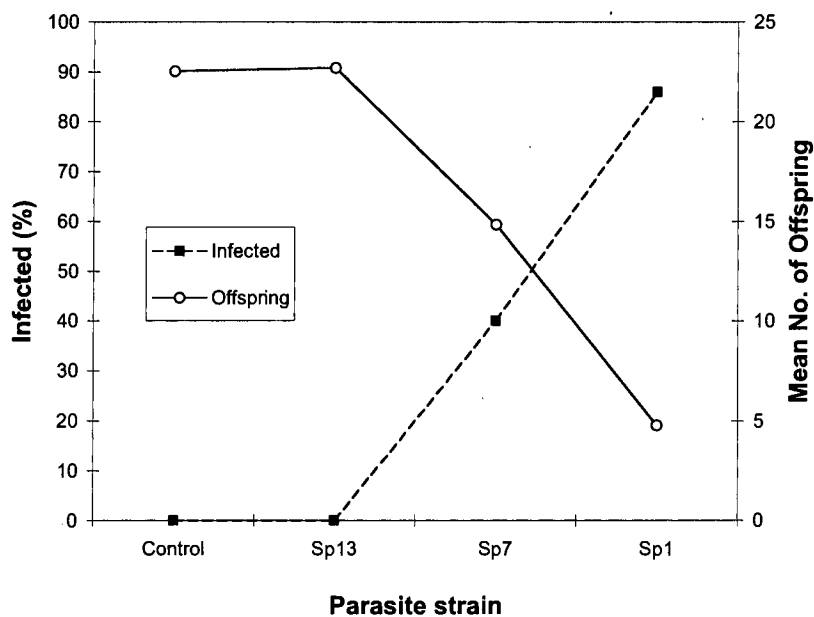


Figure 4.1. Infectivity, and its consequences on offspring production, of the three *Pasteuria ramosa* strains used in this study, compared to uninfected controls.

4.3.2. Experimental design

The experiment was divided into a primary exposure phase, secondary exposure phase and post-infection phase. All of the replicates were established using 2<sup>nd</sup> – 4<sup>th</sup> brood female neonates (<24hrs old) taken from the same 1.5l jar, with six *Daphnia* per

replicate. Eighty replicates were allocated to three main treatment groups, with each group corresponding to the parasite isolate that would be encountered during the primary exposure phase (No Spores [NS], Sp8 or Sp13). These were then divided into four subgroups corresponding to the parasite treatment received during the secondary exposure phase (i.e. Sp1, Sp7, Sp8 or Sp13), with twenty replicates per subgroup (total  $n = 240$  replicates). A double-control treatment, receiving no spores during either phase, was also included. Note, I could not study the case where both the initial and secondary exposures involved the virulent forms of *P. ramosa* because with this combination it is not possible to determine the stage at which infection occurred, i.e. a 'Sp7/Sp1' or 'Sp1/Sp7' treatment would tell us little about how the secondary exposure was being affected, as it is not currently possible to distinguish between different strains *in vivo*.

During both exposure phases the animals were kept in 50ml jars containing *Daphnia* medium, a small quantity of sand and 1ml of the appropriate spore solution (Controls received 1ml of *Daphnia* water). They were maintained at 20°C in a 12:12 L:D cycle, and were fed  $5 \times 10^5$  algal cells *Daphnia*<sup>-1</sup> day<sup>-2</sup>. Jars were randomly allocated to trays (24 jars per tray) and both jars and trays were rotated daily to minimise positional effects. Each exposure lasted five days with the animals being moved to fresh jars for the secondary exposure phase, which followed immediately after the primary exposure phase.

For the post-infection phase, the animals were moved to 200ml jars and feeding was increased to  $3 \times 10^6$  algal cells *Daphnia*<sup>-1</sup> day<sup>-2</sup>. Again, jars and trays were rotated daily (12 jars per tray) to minimise positional effects. Medium was changed every other day and the number of adults and offspring (if present) were recorded. The post-infection



phase lasted for 21 days, at the end of which the number of infected and uninfected females was recorded.

#### **4.3.3. Analysis**

Data from the post-infection phase was used to assess the effects of each treatment. The proportion of individuals infected was analysed with logistic regression and the total mean number of (square-root+0.5 transformed) offspring produced per replicate were analysed by two-way ANOVA. The main effects of “primary exposure” and “secondary exposure” were tested, as was the interaction between them; the significance of the interaction term would indicate whether the effects of a strain encountered during the SE phase was dependent on the particular strain encountered during the PE phase. All analyses were performed with SAS procedure GENMOD and differences between strains were analysed through the ‘contrast’ statement.

#### **4.4. Results**

Mean offspring production and mean infection levels across treatments are shown in figures 4.2 and 4.3, respectively. Before I carried out the analysis, I removed the ‘NS/NS’, ‘Sp8/Sp8’, ‘Sp13/Sp13’, ‘Sp8/Sp13’, ‘Sp13/Sp8’, ‘NS/Sp8’ and ‘NS/Sp13’ groups from the data set. The main point of including these treatments in the experimental design was to: (a) show that strains Sp8 and Sp13 were indeed essentially unable to infect this host clone; (b) rule out the possibility that any effects on infection

levels or offspring production were simply due to some treatments receiving a double-dose of spores (as compared to those only receiving a dose in the secondary exposure phase). These exposures had no impact on offspring production (One-way ANOVA, treatment group as fixed effect;  $F_{6, 120} = 0.417, P = 0.867$ ). However, infection levels across these treatments were negligible (0 - <3%), so sample sizes of 17 – 20 per treatment would not have had the power to detect double-dose effects if they had occurred.

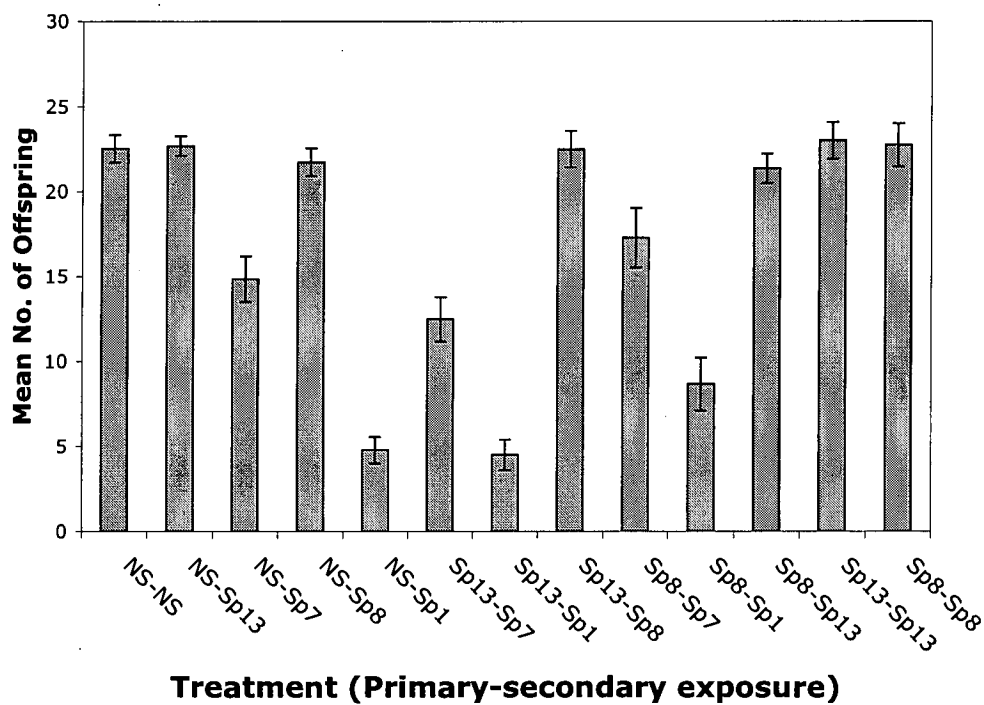
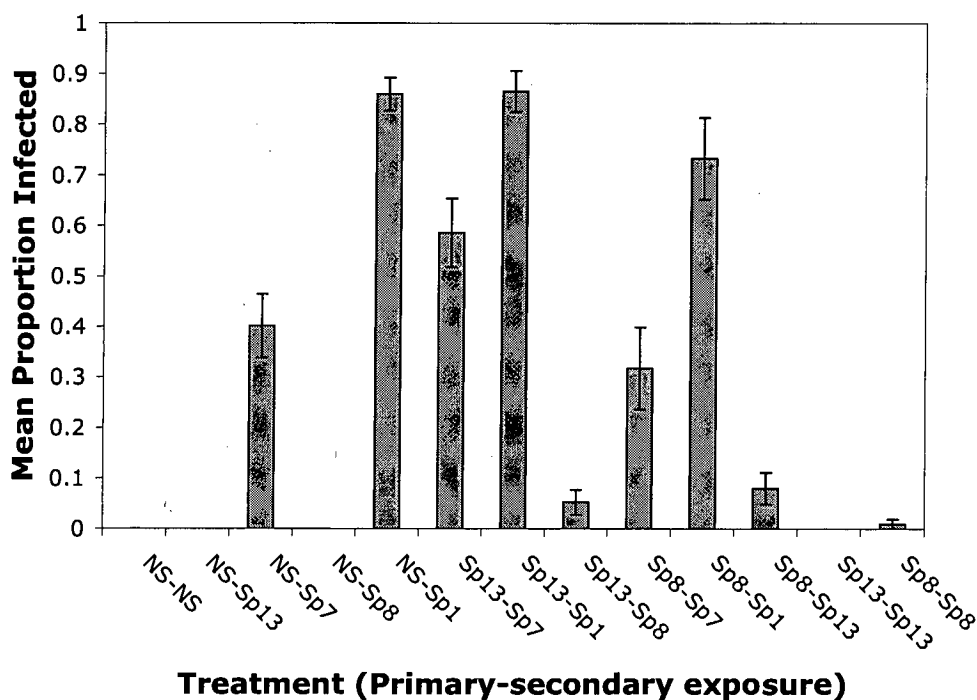


Figure 4.2. Mean offspring production across the thirteen exposure combinations. NS denotes ‘No Spores’. Data are untransformed for clarity, bars represent the standard error.



**Figure 4.3.** Mean infection levels across the thirteen exposure combinations. NS denotes 'No Spores'. Data are untransformed for clarity, bars represent the standard error.

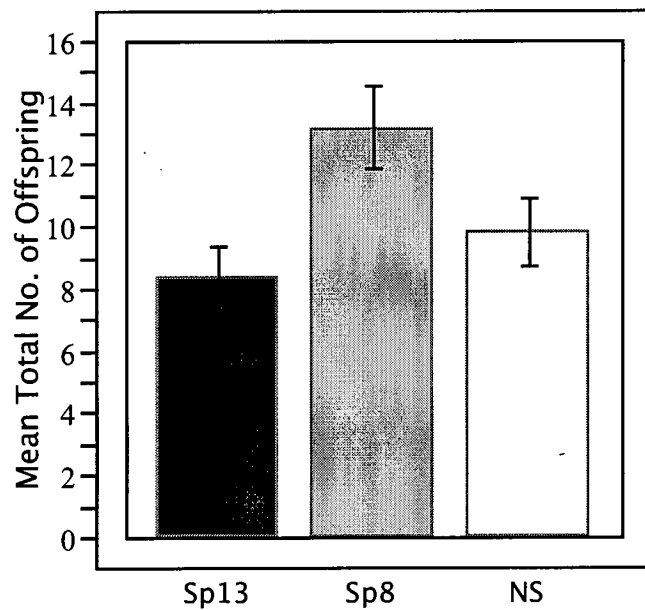
Both primary and secondary exposure significantly impacted both the proportion of hosts becoming infected and offspring production, but no interaction was found between the two exposures (Table 4.1). Post-hoc contrasts indicate that the significant effect of the primary exposure is attributable to differences between the Sp8 and the no spores (NS) treatments, and the Sp8 and Sp13 treatments. The Sp13 treatment did not differ from the NS treatment. Specifically, lines initially exposed to strain Sp8 showed significantly lower infection levels than those primarily exposed to Sp13 of those that experienced no spores (Figs. 4.4 & 4.5; Table 4.2).

<b><u>Infection level</u></b>			
	<u>N/D d.f.</u>	<u>L.R. <math>\chi^2</math></u>	<u>P</u>
<i>Primary Exposure</i>	2, 109	10.53	0.005
<i>Secondary Exposure</i>	1, 109	79.96	<0.0001
<i>P.E. x S.E.</i>	2, 109	3.45	0.179

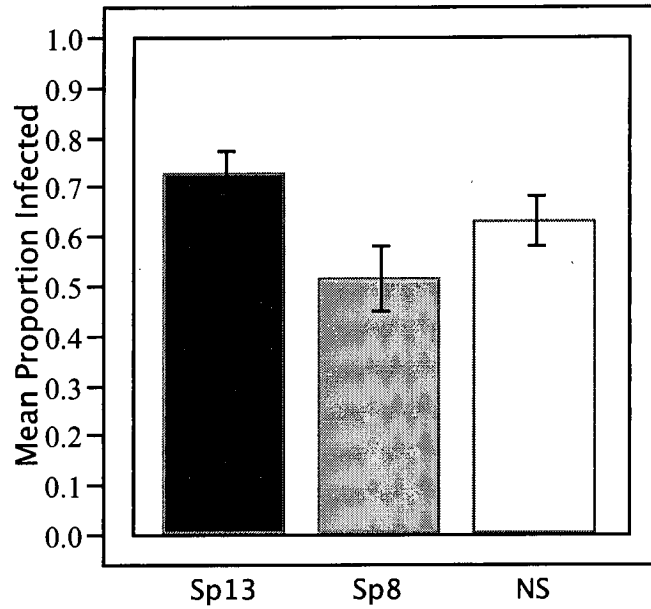
  

<b><u>Offspring production</u></b>			
	<u>N/D d.f.</u>	<u>F</u>	<u>P</u>
<i>Primary Exposure</i>	2, 109	4.11	0.019
<i>Secondary Exposure</i>	1, 109	61.70	<0.0001
<i>P.E. x S.E.</i>	2, 109	0.34	0.711

**Table 4.1.** ANOVA table detailing the effects of primary exposure, secondary exposure and their interaction. N/D are the numerator and denominator degrees of freedom.



**Figure 4.4.** Comparison of the effect of the primary exposure strains on offspring production. Data are untransformed for clarity, bars represent the standard error.

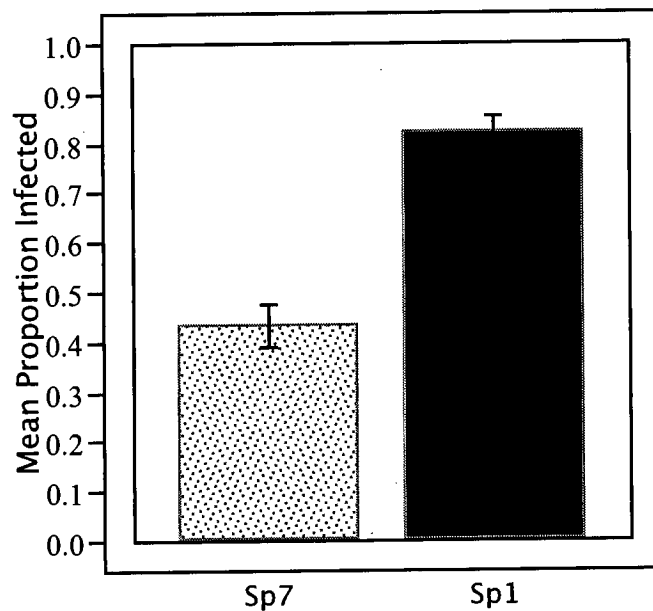


**Figure 4.5.** Comparison of the effect of the primary exposure strains on infection levels. Data are untransformed for clarity, bars represent the standard error.

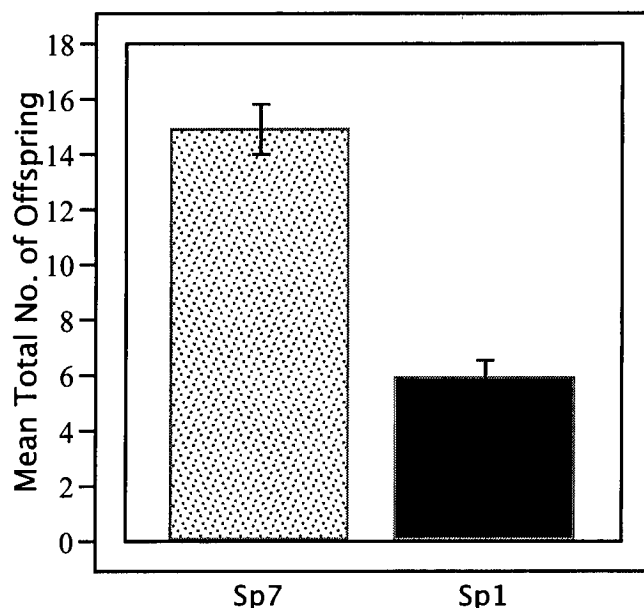
<u>Infection level</u>			
<u>Contrast</u>	<u>N/D d.f.</u>	<u>L.R. <math>\chi^2</math></u>	<u>P</u>
<i>Control</i> vs. <i>Sp8</i>	1, 109	5.59	0.018
<i>Control</i> vs. <i>Sp13</i>	1, 109	0.75	0.388
<i>Sp13</i> vs. <i>Sp8</i>	1, 109	10.11	0.002
<u>Offspring production</u>			
<u>Contrast</u>	<u>N/D d.f.</u>	<u>F</u>	<u>P</u>
<i>Control</i> vs. <i>Sp8</i>	1, 109	2.95	0.089
<i>Control</i> vs. <i>Sp13</i>	1, 109	1.25	0.265
<i>Sp13</i> vs. <i>Sp8</i>	1, 109	8.11	0.005

**Table 4.2.** Results of LS means contrast analysis comparing infection levels and offspring production amongst the three primary exposure treatments groups. N/D are the numerator and denominator degrees of freedom.

The significant effect of secondary exposure is simply due to the fact that Sp1 is more infective ( $F_{1,109} = 47.69, P < 0.0001$ ; Fig. 4.5) and causes larger fecundity reduction ( $F_{1,109} = 62.0, P < 0.0001$ ; Fig. 4.6) than does Sp7. The non-significant interaction between primary and secondary exposure suggests that although primary exposure did influence later infection, that influence did not depend on the subsequent strain (Sp1 or Sp7).



**Figure 4.6.** Comparison of the effect of the secondary exposure strains on infection levels. Data are untransformed for clarity, bars represent the standard error.



**Figure 4.6. Comparison of the effect of the secondary exposure strains on offspring production. Data are untransformed for clarity, bars represent the standard error.**

#### 4.5. Discussion

This study found that prior exposure of *D. magna* to avirulent strains of *P. ramosa* (Sp8 & Sp13) can influence the outcome of later exposure and infection. The type and magnitude of the effect, however, was entirely dependent on the strain encountered in the primary exposure; prior exposure to Sp8 actually reduced later infection while prior exposure to Sp13 had no effect on later infection. Thus, prior exposure could have either beneficial or neutral effects on subsequent infection. I also found that both single and repeat exposures to strains Sp8 and Sp13 had no effect on offspring production (as observed in the seven treatments removed from the final analysis), suggesting that there are no obvious costs associated with resistance to these two strains. Although the sample

sizes used here would not have allowed me to detect a double-dose effect on infection levels, double-dose effects can probably be ruled out as a potential explanation for the patterns observed in this study; an increase in infection levels, due to an increase in the number of spores the hosts were exposed to, would have been the predicted effect under such circumstances, which is inconsistent with the pattern of results.

Costs of resistance have been identified in a wide range of organisms (reviewed in Kraaijeveld *et al.*, 2002), and they can be broken down into two broad categories. Firstly, the costs of maintaining resistance are those that apply to aspects of the immune system being maintained in anticipation of a pathogen attack. For example, higher levels of resistance to parasitoids in *Drosophila melanogaster* are associated with reduced levels of larval competitive ability (Kraaijeveld & Godfray, 1997; Fellowes *et al.*, 1998), and this appears to be due to a trade-off between increased investment in the number of haemocytes circulating in the blood (Eslin & Prévost, 1998; Kraaijeveld *et al.*, 2001) and larval feeding rate (Fellowes *et al.*, 1999<sup>a</sup>). Secondly, there are the costs of induced defence resulting from the deployment of the immune response following parasitism. Again, using *D. melanogaster* as an example, it has been shown that larvae successful in encapsulating a parasitoid egg suffer from reduced body size and fecundity as adults (Fellowes *et al.*, 1999<sup>b</sup>), and this is thought to be due to the reallocation of resources from growth and feeding to defence (Kraaijeveld *et al.*, 2002).

At present, there is no evidence that immunity to *P. ramosa* infection is costly in *D. magna*. A comparison of life-history traits and competitive ability in clones broadly resistant and broadly susceptible to *P. ramosa* infection by Little *et al.* (2002) found no evidence for standing costs of resistance. In this study, I found that exposure to avirulent



strains had no impact on fecundity, suggesting that costs of induced defence are physiologically inconsequential, or can only be observed under conditions of stress (Kraaijeveld *et al.*, 2002).

However, this conclusion rests on the assumption that avirulence is due to the host actively resisting infection by these strains, which does not entirely fit with the infection patterns observed here. If active resistance was involved then lines that had been 'primed' by initial exposure to both strains Sp8 and Sp13 would have been expected to have shown lower infection rates when exposed to the more virulent strains. Although there is some evidence that *D. magna* may be able to produce defence molecules that are effective against specific *P. ramosa* strains (Little *et al.*, 2003), it seems unlikely that the hosts would not also launch a much more general immune response at the same time. Even if this did not completely immunise the hosts against further attack, it would at least be expected to make invasion by subsequent strains more difficult. However, lines primarily exposed to strain Sp13 did not differ in infection level from the unprimed control treatment, suggesting that no significant general immune reaction had been triggered. The significant difference between the Sp8- and Sp13-primed treatments is interesting, as it again highlights some of the complex genotypic interactions underlying the resistance patterns observed in this system (Carius *et al.*, 2001; Mitchell *et al.*, 2005). Prior exposure to Sp8 did have an immunising effect, which suggests that this strain may share some properties with the two more virulent strains. In this respect, effector molecules launched against specific Sp8 could potentially also have an inhibitory effect on strains Sp7 and Sp1.

Of course, the basis of resistance may not just lie in hosts' ability to actively resist infection, it could be also be due to a parasites inability to infect specific hosts. Little *et al.* (2005) have suggested that specificity may arise not just from variation in immune system components but from variation in structures and molecules exploited by parasites for invasion or replication (e.g. cell-surface binding proteins). Since infection in this system occurs through the uptake of infective spores from the environment (Ebert *et al.*, 1996) it is entirely plausible that variation in the structure of cell surface proteins in the host gut, and corresponding variation in binding structures on the bacterial cell surface, may be responsible for the genotype-specific infection patterns observed. If this is the case then infection genetics in this system may resemble the 'matching-alleles' model of resistance, where a specific match between host genotype and parasite genotype is required for infection to occur (Hamilton *et al.*, 1990; Agrawal & Lively, 2002).

One further possibility is that Sp8 did manage to infect the hosts, albeit at undetectable levels, and by doing so it interfered competitively with strains Sp7 and Sp1. Although there is little evidence that *P. ramosa* infections are ever maintained at such low levels, it is possible that very low numbers of Sp8 were growing in host tissues and that this had an inhibitory effect on subsequent strains, either through direct competition for resources or through the release of inhibitory proteins (Riley & Gordon, 1999). Such competition is likely to occur in natural populations, as hosts in a single pond may be exposed to a variety of *P. ramosa* strains, many of which may be capable of infecting similar host genotypes (Carius *et al.*, 2001). In this respect, the difference in 'immunising' ability between strains Sp8 and Sp13 may simply be due to Sp8 being better at invading host tissues.

Despite evidence in support of an adaptive, specific invertebrate immune response, the precise nature of this specificity has yet to be ascertained. The recent discovery in two separate invertebrate groups of mechanisms able to generate high levels of diversity in Ig-superfamily proteins (Zhang *et al.*, 2004; Watson *et al.*, 2005) highlights the potential for a significant level of specificity at the recognition stage. However, the discriminatory ability of the majority of immune system-related recognition proteins currently identified – commonly referred to as pattern-recognition receptors (PRPs) – appears to function at a very general level; they have so far only been shown to be able to distinguish between the likes of Gram-negative and Gram-positive bacteria, or between bacterial and fungal infections (Leimatre *et al.*, 1997; Michel *et al.*, 2001; Ferrandon *et al.*, 2003; Little *et al.*, 2004). Likewise, the humoral and cellular effector mechanisms (e.g. AMP production, encapsulation, phagocytosis) are effective against a very broad range of pathogens, and there is currently little evidence that any of the molecules or compounds produced during an immune reaction function at a highly specific level (Carton & Nappi, 1997; Bulet *et al.*, 1999; Schmid-Hempel, 2005). At present little is known about how different *P. ramosa* isolates vary at either a genetic or structural level, and further investigation into the sources of variation between strains is required to shed more light onto the workings of these interactions.

## Chapter 5. General Discussion

### Long live the Red Queen?

The Red Queen hypothesis postulates that antagonistic coevolution between parasites and their hosts is responsible for the evolutionary maintenance of sex (Bell, 1982; Hamilton *et al.*, 1990). On the surface, it is easy to see why the Red Queen is such an attractive proposition; parasites are ubiquitous in nature and as such they are likely to be a constant source of selection in most species. The work presented in this thesis, however, highlights some of the Red Queen's inadequacies, in particular the complex genetic and environmental interactions that underlie many, if not all, host-parasite relationships. At present, I very much doubt that advocates of the Red Queen hypothesis will be able to resolve its various deficiencies. Of particular concern is the increasing body of work demonstrating that genetic interactions between hosts and parasites can be reversed, or even rendered obsolete, by fluctuations in temperature (Chapter 3; Thomas & Blanford, 2003; Mitchell *et al.*, 2005); this is potentially disastrous for a hypothesis that relies so heavily upon genotype-specific infection patterns. Recent studies uncovering evidence of specific immunological memory in the invertebrate immune system (Chapter 4; Little *et al.*, 2005; Schmid-Hempel, 2005) are yet another potential nail in the coffin.

As was discussed in the opening chapter, most studies attempt to test the predictions of the Red Queen by either, (i) examining the relationship between host genotype frequency and infection by parasites, (ii) comparing levels of infection in closely related sexual and asexual species, or (iii) looking for a relationship between

levels of infection and levels of sex and recombination within species. These studies across a range of taxa have not entirely concurred, and this is not surprising, given that very few of them acknowledge the intricate genotype-by-genotype-by-environment interactions that are likely to play a significant role in many of these systems. Ideally, not only should new studies make the effort to incorporate as many of the above approaches as possible (e.g. Lively, 1990; Michiels *et al.* 2001; Jokela *et al.*, 2003), they should also attempt to examine the interactions influencing patterns of resistance and virulence in natural populations. Only then will it be possible to draw informed conclusions on the Red Queen's role in the evolutionary maintenance of sexual reproduction.

## References

- Agrawal, A.F. (2001) Sexual selection and the maintenance of sexual reproduction. *Nature* **411**: 692-695.
- Agrawal, A. and Lively, C.M. (2002) Infection genetics: gene-for-gene versus matching-alleles models and all points in between. *Evolutionary Ecology Research* **4**: 79-90.
- Aparici, E., Carmona, M.J. and Serra, M. (2002) Evidence for an even sex allocation in haplodiploid cyclical parthenogens. *Journal of Evolutionary Biology* **15**: 65-73.
- Arala-Chaves, M. and Sequeira, T. (2000) Is there any kind of adaptive immunity in invertebrates? *Aquaculture* **191**: 247-258.
- Barnes, P.T., Holland, B. and Courreges, V. (1989) Genotype-by-environment and epistatic interactions in *Drosophila melanogaster*: The effects of *Gpdh* allozymes, genetic background and rearing temperature on larval development time and viability. *Genetics* **122**: 859-868.
- Barton, N.H. (1995) A general model for the evolution of recombination. *Genetical Research* **65**: 123-144.

Barton, N.H. and Charlesworth, B. (1998) Why sex and recombination? *Science* **281**: 1986-1989.

Bell, G. (1982) *The Masterpiece of Nature: The Evolution and Genetics of Sexuality*. University of California Press, Berkley.

Ben-Ami, F. and Heller, J. (2005) Spatial and temporal patterns of parthenogenesis and parasitism in the freshwater snail *Melanoides tuberculata*. *Journal of Evolutionary Biology* **18**: 138-146.

Berg, L.M., Pálsson, S. and Lascoux, M. (2001) Fitness and sexual response to population density in *Daphnia pulex*. *Freshwater Biology* **46**: 667-677.

Bierzychudek, P. (1985) Patterns in plant parthenogenesis. *Experientia* **41**: 1255-1264.

Blanford, S., Thomas, M.B., Pugh, C. and Pell, J.K. (2003) Temperature checks the Red Queen? Resistance and virulence in a fluctuating environment. *Ecology Letters* **6**: 2-5.

Brown, S.G., Kwan, S. and Shero, S. (1995) The parasitic theory of sexual reproduction: parasitism in unisexual and bisexual geckos. *Proceedings of the Royal Society of London, Series B* **260**: 317-320.

Browne, R.A. and MacDonald, G.H. (1982) Biogeography of the brine shrimp, *Artemia*: distribution of parthenogenetic and sexual populations. *Journal of Biogeography* **9**: 331-338.

Buckling, A., Wei, Y., Massey, R.C., Brockhurst, M.A. and Hochberg, M.E. (2006) Antagonistic coevolution with parasites increases the cost of host deleterious mutations. *Proceedings of the Royal Society of London, series B* **273**: 45-49.

Bulet, P., Hetru, C., Dimarcq, J.-L. and Hoffmann, D. (1999) Antimicrobial peptides in insects; structure and function. *Developmental and Comparative Immunology* **23**: 329-344.

Burt, A. (2000) Sex, recombination and the efficacy of selection – was Weissman right? *Evolution* **54**: 337-351.

Camacho, J.P.M., Bakkali, M., Corral, J.M., Cabrero, J., López-León, M.D., Aranda, I., Martin-Alganza, A. and Perfectti, F. (2002) Host recombination is dependent on the degree of parasitism. *Proceedings of the Royal Society of London, Series B* **269**: 2173-2177.

Camara, M.D., Ancell, C.A. and Pigliucci, M. (2000) Induced mutations: A novel tool to study phenotypic integration and evolutionary constraints in *Arabidopsis thaliana*. *Evolutionary Ecology Research* **2**: 1009-1029.



- Carius, H.J., Little, T.J. and Ebert, D. (2001) Genetic variation in a host-parasite association: potential for coevolution and frequency-dependent selection. *Evolution* **55**: 1136-1145.
- Carton, Y. and Nappi, A.J. (1997) *Drosophila* cellular immunity against parasitoids. *Parasitology Today* **13**: 218-227.
- Carvalho, G.R. and Crisp, D.J. (1987) The clonal ecology of *Daphnia magna* (Crustacea: Cladocera). I. Temporal changes in the clonal structure of a natural population. *Journal of Animal Ecology* **56**: 453-468.
- Castle, W.E. (1921) An improved method of estimating the number of genetic factors concerned in cases of blending inheritance. *Science* **54**: 223.
- Charlesworth, B. (1990) Mutation-selection balance and the evolutionary advantage of sex and recombination. *Genetical Research* **55**: 199-221.
- Cooper, T.F., Lenski, R.E. and Elena, S.F. (2005) Parasites and mutational load: an experimental test of a pluralist theory for the evolution of sex. *Proceedings of the Royal Society of London, Series B* **272**: 311-317.

- Corley, L.S., Blankenship, J.R. and Moore, A.J. (2001) Genetic variation and asexual reproduction in the facultatively parthenogenetic cockroach *Nauphoeta cinerea*: implications for the evolution of sex. *Journal of Evolutionary Biology* **14**: 68-74.
- Crease, T.J., Stanton, D.J. and Hebert, P.D.N. (1989) Polyphyletic origins of asexuality in *Daphnia pulex*. II. Mitochondrial-DNA variation. *Evolution* **43**: 1016-1026.
- Davies, E.K., Peters, A.D. and Keightley, P.D. (1999) High frequency of cryptic deleterious mutations in *Caenorhabditis elegans*. *Science* **285**: 1748-1751.
- De La Pena, M., Elena, S.F. and Moya, A. (2000) Effect of deleterious mutation-accumulation on the fitness of RNA bacteriophage MS2. *Evolution* **54**: 686-691.
- De Meester, L. and Vanoverbeke, J. (1999) An uncoupling of male and sexual egg production leads to reduced inbreeding in the cyclical parthenogen *Daphnia*. *Proceedings of the Royal Society of London, Series B* **266**: 2471-2477.
- De Mott, W.R. (1982) Feeding selectivities and relative ingestion rates of *Daphnia* and *Bosmina*. *Limnology and Oceanography* **27**: 518-527.
- De Visser, J.A.G.M. and Rozen, D.E. (2006) Clonal interference and the periodic selection of new beneficial mutations in *Escherichia coli*. *Genetics* **172**: 2093-2100.

De Visser, J.A.G.M., Hoekstra, R.F. and Van Den Ende, H. (1996) The effect of sex and deleterious mutations on fitness in *Chlamydomonas*. *Proceedings of the Royal Society of London, Series B* **263**: 193-200.

De Visser, J.A.G.M., Hoekstra, R.F. and Van Den Ende, H. (1997) An experimental test for synergistic epistasis and its application in *Chlamydomonas*. *Genetics* **145**: 815-819.

Decaestecker, E., Vergote, A., Ebert, D. and De Meester, L. (2003) Evidence for strong host clone-parasite species interactions in the *Daphnia* microparasite system. *Evolution* **57**: 784-792.

Deng, H.-W. and Lynch, M. (1997) Inbreeding depression and inferred deleterious mutation parameters in *Daphnia*. *Genetics* **147**: 147-155.

Drake, J.W., Charlesworth, B., Charlesworth, D. and Crow, J.F. (1998) Rates of spontaneous mutation. *Genetics* **148**: 1667-1686.

Duncan, A., Mitchell, S.E. and Little, T.J. (2006) Parasite-mediated selection and the role of sex and diapause in *Daphnia*. *Journal of Evolutionary Biology*. In Press.

Dybdahl, M.F. and Lively, C.M. (1998) Host-parasite coevolution: evidence for rare advantage and time-lagged selection in a natural population. *Evolution* **52**: 1057-1066.

Ebert, D. (1995) The ecological interactions between a microsporidian parasite and its host *Daphnia magna*. *Journal of Animal Ecology* **64**: 361-369.

Ebert, D., Rainey, P., Embley, T.M. and Scholz, D. (1996) Development, life cycle, ultrastructure and phylogenetic position of *Pasteuria ramosa* Metchnikoff 1888: rediscovery of an obligate endoparasite of *Daphnia magna* Strauss. *Philosophical Transactions of the Royal Society of London, Series B* **351**: 1689-1701.

Ebert, D., Zschokke-Rohringer, C.D. and Carius, H.J. (1998) Within- and between-population variation for resistance of *Daphnia magna* to the bacterial endoparasite *Pasteuria ramosa*. *Proceedings of the Royal Society of London, Series B* **265**: 2127-2134.

Elena, S.F. and de Visser, J.A.G.M. (2003) Environmental stress and the effects of mutation. *Journal of Biology* **2**: 12.

Elena, S.F. and Lenski, R.E. (1997) Test of synergistic interactions among deleterious mutations in bacteria. *Nature* **390**: 395-397.

Fellowes, M.D.E., Kraaijeveld, A.R. and Godfray, H.C.J. (1998) Trade-off associated with selection for increased ability to resist parasitoid attack in *Drosophila melanogaster*. *Proceedings of the Royal Society of London, series B* **265**: 1553-1558.

Fellowes, M.D.E., Kraaijeveld, A.R. and Godfray, H.C.J. (1999)a Association between feeding rate and parasitoid resistance in *Drosophila melanogaster*. *Evolution* **53**: 1302-1305.

Fellowes, M.D.E., Kraaijeveld, A.R. and Godfray, H.C.J. (1999)b The relative fitness of *Drosophila melanogaster* (Diptera, Drosophilidae) that have successfully defended themselves against the parasitoid *Asobara tabida* (Hymenoptera, Braconidae). *Journal of Evolutionary Biology* **12**: 123-128.

Fisher, R.A. (1930) *The Genetical Theory of Natural Selection*. Oxford University Press, Oxford.

Frank, S.A. (1987) Individual and population sex allocation patterns. *Theoretical Population Biology* **31**: 47-74.

Frank, S.A. (1990) Sex allocation theory for birds and mammals. *Annual Review of Ecology and Systematics* **21**: 13-55.

Fry, J.D. (2004) On the rate and linearity of viability declines in *Drosophila* mutation-accumulation experiments: Genomic mutation rates and synergistic epistasis revisited. *Genetics* **166**: 797-806.

Fry, J.D., Keightley, P.D., Heinsohn, S.L. and Nuzhdin, S.V. (1999) New estimates of the rates and effects of mildly deleterious mutation in *Drosophila melanogaster*.

*Proceedings of the National Academy of Sciences, USA* **96**: 574-579.

Geedey, C.K., Tessier, A.J. and Machledt, K. (1996) Habitat heterogeneity, environmental change, and the clonal structure of *Daphnia* populations. *Functional Ecology* **10**: 613-621.

Giebelhausen, B. and Lampert, W. (2001) Temperature reaction norms of *Daphnia magna*: the effect of food concentration. *Freshwater Biology* **13**: 371-382.

Glesener, R.R. and Tilman, D. (1978) Sexuality and the components of environmental uncertainty: clues from geographic parthenogenesis in terrestrial animals. *American Naturalist* **112**: 659-673.

Green, J. (1974) Parasites and epibionts of Cladocera. *Transactions of the Zoological Society of London* **32**: 417-515.

Haag, C.R., Sakwinska, O. and Ebert, D. (2003) Test of synergistic interactions between infection and inbreeding in *Daphnia magna*. *Evolution* **57**: 777-783.

Hadany, L. and Feldman, M.W. (2005) Evolutionary traction: the cost of adaptation and the evolution of sex. *Journal of Evolutionary Biology* **18**: 309-314.

Hakoyama, H., Nishimura, T., Matsubara, N. and Iguchi, K. (2001) Difference in parasite load and nonspecific immune reaction between sexual and gynogenetic forms of *Carassius auratus*. *Biological Journal of the Linnaean Society* **72**: 401-407.

Hamilton, W.D., Axelrod, R. and Tanese, R. (1990) Sexual reproduction as an adaptation to resist parasites (A Review). *Proceedings of the National Academy of Sciences, USA* **87**: 3566-3573.

Hanley, K.A., Fisher, R.N. and Case, T.J. (1995) Lower mite infestations in an asexual gecko compared with its sexual ancestors. *Evolution* **49**: 418-426.

Hartl, D.L. and Jones, E.W. (1999) *Essential Genetics*; 2<sup>nd</sup> edition. Jones and Bartlett Publishers, Sudbury, MA.

Hastings, I.M. (1999) The costs of sex due to deleterious intracellular parasites. *Journal of Evolutionary Biology* **12**: 177-183.

Hebert, P.D.N. (1978) The population of biology of *Daphnia* (Crustacea, Daphnidae). *Biological Review* **53**: 387-426.

Hebert, P.D.N. (1981) Obligate asexuality in *Daphnia*. *American Naturalist* **117**: 784-789.

Hebert, P.D.N. (1987) Genotypic characteristics of cyclic parthenogens and their obligately asexual derivatives. In, *The evolution of sex and its consequences* (Stearns, S.J., ed.), pp. 175-195, Birkhauser Verlag.

Hebert, P.D.N. and Ward, R.D. (1976) Enzyme variability in natural populations of *Daphnia magna* IV. Ecological differentiation and frequency changes of genotypes at Audley End. *Heredity* **36**: 331-341.

Hebert, P.D.N. and Crease, T. (1983) Clonal diversity in populations of *Daphnia pulex* reproducing by obligate parthenogenesis. *Heredity* **51**: 353-369.

Hebert, P.D.N. and Beaton, M.J. (1993) *Methodologies for allozyme analysis using cellulose acetate electrophoresis*. Helena Laboratories, Beaumont, TX.

Hebert, P.D.N. and Finston, T.L. (2001) Macrogeographic patterns of breeding system diversity in the *Daphnia pulex* group from the United States and Mexico. *Heredity* **87**: 153-161.



Hebert, P.D.N., Ward, R.D. and Weider, L.J. (1988) Clonal-diversity patterns and breeding system variation in *Daphnia pulex*, an asexual-sexual complex. *Evolution* **42**: 147-159.

Hebert, P.D.N., Beaton, M.J., Schwartz, S.S. and Stanton, D.J. (1989) Polyphyletic origins of asexuality in *Daphnia pulex*. I. Breeding system variation and levels of clonal diversity. *Evolution* **43**: 1004-1015.

Hebert, P.D.N., Schwartz, S.S., Ward, R.D. and Finston, T.L. (1993) Macrogeographic patterns of breeding system diversity in the *Daphnia pulex* group. I. Breeding systems of Canadian populations. *Heredity* **70**: 148-161.

Heller, J. and Farstey, V. (1990) Sexual and parthenogenetic populations of the freshwater snail *Melanooides tuberculata* in Israel. *Israel Journal of Zoology* **37**: 75-87.

Hitotsumachi, S., Carpenter, D.A. and Russell, W.L. (1985) Dose-repetition increases the mutagenic effectiveness of *N*-ethyl-*N*-nitrosourea in mouse spermatogonia. *Proceedings of the National Academy of Sciences, USA* **82**: 6619-6621.

Holland, B. and Rice, W.R. (1999) Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. *Proceedings of the National Academy of Sciences, USA* **96**: 5083-5088.

Howard, R.S. and Lively, C.M. (1994) Parasitism, mutation accumulation and the maintenance of sex. *Nature* **367**: 554-557.

Howard, R.S. and Lively, C.M. (1998) The maintenance of sex by parasitism and mutation accumulation under epistatic fitness functions. *Evolution* **52**: 604-610.

Howard, R.S. and Lively, C.M. (2003) Opposites attract? Mate choice for parasite evasion and the evolutionary stability of sex. *Journal of Evolutionary Biology* **16**: 681-689.

Innes, D.J. (1991) Geographic patterns of genetic differentiation among sexual populations of *Daphnia pulex*. *Canadian Journal of Zoology* **69**: 995-1003.

Innes, D.J. (1997) Sexual reproduction of *Daphnia pulex* in a temporary habitat. *Oecologia* **111**: 53-60

Innes, D.J. and Hebert, P.D.N. (1988) The origin and genetic basis of obligate parthenogenesis in *Daphnia pulex*. *Evolution* **42**: 1024-1035.

Innes, D.J. and Singleton, D.R. (2000) Variation in allocation to sexual and asexual reproduction among clones of cyclically parthenogenetic *Daphnia pulex* (Crustacea: Cladocera). *Biological Journal of the Linnean Society* **71**: 771-787.

Innes, D.J., Fox, C.J. and Winsor, G.L. (2000) Avoiding the cost of males in obligately asexual *Daphnia pulex* (Leydig). *Proceedings of the Royal Society of London, Series B* **267**: 991-997.

Innes, D.J., Schwartz, S.S. and Hebert, P.D.N. (1986) Genotypic diversity and variation in mode of reproduction among populations in the *Daphnia pulex* group. *Heredity* **57**: 345-355.

Johnson, S.G. (2000) Population structure, parasitism, and survivorship of sexual and autodiploid parthenogenetic *Campeloma limum*. *Evolution* **54**: 167-175.

Jokela, J. and Lively, C.M. (1995) Parasites, sex, and early reproduction in a mixed population of freshwater snails. *Evolution* **49**: 1268-1271.

Jokela, J., Lively, C.M., Dybdahl, M.F. and Fox, J. (1997) Evidence for a cost of sex in the freshwater snail *Potamopyrgus antipodarum*. *Evolution* **78**: 452-460.

Jokela, J., Lively, C.M., Dybdahl, M.F. and Fox, J.A. (2003) Genetic variation in sexual and clonal lineages of a freshwater snail. *Biological Journal of the Linnaean Society* **79**: 165-181.

Justice, M.J., Carpenter, D.A., Favor, J., Neuhauser-Klaus, A., Hrabé de Angelis, M., Soewarto, D., Moser, A., Cordes, S., Miller, D., Chapman, V., Weber, J.S., Rinchik,

E.M., Hunsicker, P.R., Russell, W.L. and Bode, V.C. (2000) Effects of ENU dosage on mouse strains. *Mammalian Genome* **11**: 484-488.

Keightley, P.D. and Eyre-Walker, A. (2000) Deleterious mutations and the evolution of sex. *Science* **290**: 331-333.

Keightley, P.D. and Eyre-Walker, A. (2001) Response to Kondrashov. *Trends in Genetics* **17**: 77-78.

Kelley, S.E. (1994) Viral pathogens and the advantage of sex in the perennial grass *Anthoxanthum odoratum*. *Philosophical Transactions of the Royal Society of London, Series B* **346**: 295-302.

Kelley, S.E., Antonovics, J. and Schmitt, J. (1988) A test of the short-term advantage of sexual reproduction. *Nature* **331**: 714-716.

Killick, S.C., Carlsson, A.M., West, S.A. and Little, T.J. (2006) Testing the pluralist approach to sex: the influence of environment on synergistic interactions between mutation load and parasitism in *Daphnia magna*. *Journal of Evolutionary Biology*. In Press.

Kishony, R. and Leibler, S. (2003) Environmental stresses can alleviate the average deleterious effect of mutations. *Journal of Biology* **2**: 14.

Klein, J. (1997) Homology between immune responses in vertebrates and invertebrates: does it exist? *Scandinavian Journal of Immunology* **46**: 558-564.

Kleiven, O.T., Larsson, P. and Hobaek, A. (1992) Sexual reproduction in *Daphnia magna* requires three stimuli. *Oikos* **65**: 197-206.

Klüttgen, B.U., Dülmer, U., Engels, M. and Ratte, H.T. (1994) ADaM, an artificial freshwater for the culture of zooplankton. *Water Research* **28**: 743-746.

Kondrashov, A.S. (1982) Selection against harmful mutations in large sexual and asexual populations. *Genetical Research* **40**: 325-332.

Kondrashov, A.S. (1988) Deleterious mutations and the evolution of sexual reproduction. *Nature* **336**: 435-440.

Kondrashov, A.S. (1993) Classification of hypotheses on the advantage of amphimixis. *Journal of Heredity* **84**: 372-387.

Kondrashov, A.S. (2001) Sex and U. *Trends in Genetics* **17**: 75-77.

Kondrashov, A.S. and Houle, D. (1994) Genotype-environment interactions and the estimation of the genomic mutation rate in *Drosophila melanogaster*. *Proceedings of the Royal Society of London, series B* **258**: 221-227.

Korona, R. (1999) Genetic load of the yeast *Saccharomyces cerevisiae* under diverse environmental conditions. *Evolution* **53**: 1966-1971.

Korpelainen, H. (1986) Temporal changes in the genetic structure of *Daphnia magna* populations. *Heredity* **57**: 5-14.

Kraaijeveld, A.R. and Godfray, H.C.J. (1997) Trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. *Nature* **389**: 278-280.

Kraaijeveld, A.R., Ferrari, J. and Godfray, H.C.J. (2002) Costs of resistance in insect-parasite and insect-parasitoid interactions. *Parasitology* **125**: S71-S82.

Kramer, M.G. and Templeton, A.R. (2001) Life-history changes that accompany the transition from sexual to parthenogenetic reproduction in *Drosophila mercatorum*. *Evolution* **55**: 748-761.

Kumpulainen, T., Grapputo, A. and Mappes, J. (2004) Parasites and sexual reproduction in Psychid moths. *Evolution* **58**: 1511-1520.

Kurtz, J. (2005) Specific memory within innate immune systems. *Trends in Immunology* **26**: 186-192.

Kurtz, J. and Franz, K. (2003) Evidence for memory in vertebrate immunity. *Nature* **425**: 37-38.

Ladle, R.J. (1992) Parasites and sex: catching the Red Queen. *Trends in Ecology and Evolution* **7**: 405-408.

Lamb, R.Y. and Willey, R.B. (1979) Are parthenogenetic and related bisexual insects equal in fertility? *Evolution* **33**: 774-775.

Lambrechts, L., Halbert, J., Durand, P., Gouagna, L.C. and Koella, J.C. (2005) Host genotype by parasite genotype interactions underlying the resistance of anopheline mosquitoes to *Plasmodium falciparum*. *Malaria Journal* **4**: 3.

LaMontagne, J.M. and McCauley, E. (2001) Maternal effects in *Daphnia*: what mothers are telling their offspring and do they listen? *Ecology Letters* **4**: 64-71.

Little, T.J. (1999) *Parasite mediated selection in Daphnia*. Ph.D. diss., Zoologisches Institut, University of Basel, Switzerland.

Little, T.J. (2002) The evolutionary significance of parasitism: do parasite-driven genetic dynamics occur *ex silico*? *Journal of Evolutionary Biology* **15**: 1-9.

Little, T.J. and Ebert, D. (1999) Associations between parasitism and host genotype in natural populations of *Daphnia* (Crustacea: Cladocera). *Journal of Animal Ecology* **68**: 134-149.

Little, T.J. and Ebert, D. (2000) Sex, linkage disequilibrium and patterns of parasitism in three species of cyclically parthenogenetic *Daphnia* (Cladocera: Crustacea). *Heredity* **85**: 257-265.

Little, T.J. and Ebert, D. (2001) Temporal patterns of genetic variation for resistance and infectivity in a *Daphnia*-microparasite system. *Evolution* **55**: 1146-1152.

Little, T.J., Carius, H.-J., Sakwinska, O. and Ebert, D. (2002) Competitiveness and life-history characteristics of *Daphnia* with respect to susceptibility to a bacterial pathogen. *Journal of Evolutionary Biology* **15**: 796-802.

Little, T.J., O'Connor, B., Colegrave, N, Watt, K. and Read, A.F. (2003) Maternal transfer of strain-specific immunity in an invertebrate. *Current Biology* **13**: 489-492.

Little, T.J. and Kraaijeveld, A.R. (2004) Ecological and evolutionary implications of immunological priming in invertebrates. *Trends in Ecology and Evolution* **19**: 58-60.



Little, T.J., Colbourne, J.K. and Crease, T.J. (2004) Molecular evolution of *Daphnia* immunity genes: Polymorphism in a *Gram-Negative Binding Protein* Gene and an  $\alpha$ -2-*Macroglobulin* Gene. *Journal of Molecular Ecology* **59**: 498-506.

Little, T.J., Hultmark, D. and Read, A.F. (2005) Invertebrate immunity and the limits of mechanistic immunology. *Nature Immunology* **6**: 651-654.

Lively, C.M. (1987) Evidence from a New Zealand snail for the maintenance of sex by parasitism. *Nature* **328**: 519-521.

Lively, C.M. (2001) Trematode infection and the distribution and dynamics of parthenogenetic snail populations. *Parasitology* **123**: S19-S26.

Lively, C.M. and Dybdahl, M.F. (2000) Parasite adaptation to locally common host genotypes. *Nature* **405**: 679-681.

Lively, C.M. and Howard, R.S. (1994) Selection by parasites for clonal diversity and mixed mating. *Philosophical Transactions of the Royal Society of London, Series B* **346**: 271-281.

Lively, C.M. and Jokela, J. (2002) Temporal and spatial distributions of parasite and sex in a freshwater snail. *Evolutionary Ecology Research* **4**: 219-226.

Lively, C.M., Craddock, C. and Vrijenhoek, R.C. (1990) Red Queen hypothesis supported by parasitism in sexual and clonal fish. *Nature* **344**: 864-866.

Lively, C.M., Dybdahl, M.F., Jokela, J., Osnas, E.E. and Delph, L.E. (2004) Host sex and local adaptation by parasites in a snail-trematode interaction. *American Naturalist* **164**: S6-S18.

Lomnicki, A. and Jasienski, M. (2000) Does fitness erode in the absence of selection? An experimental test with *Tribolium*. *Journal of Heredity* **91**: 407-411.

Lynch, M. (1984) The genetic structure of a cyclical parthenogen. *Evolution* **38**: 186-203.

Lynch, M. (1987) The consequences of fluctuating selection for isozyme polymorphisms in *Daphnia*. *Genetics* **115**: 657-669.

Lynch, M., Blanchard, J., Houle, D., Kibota, T., Schultz, S., Vassilieva, L. and Willis, J. (1999) Perspective: Spontaneous deleterious mutation. *Evolution* **53**: 645-663.

Lynch, M., Bürger, R., Butcher, D. and Gabriel, W. (1993) The mutational meltdown in asexual populations. *Journal of Heredity* **84**: 339-344.

Mangin, K.L., Lipsitch, M. and Ebert, D. (1995) Virulence and transmission modes of two microsporidia in *Daphnia magna*. *Parasitology* **111**: 133-142.

Maynard Smith, J. (1978) *The Evolution of Sex*. Cambridge University Press, Cambridge.

Michiels, N.K., Beukeboom, L.W., Greeff, J.M. and Pemberton, A.J. (1999) Individual control over reproduction: an underestimated element in the maintenance of sex? *Journal of Evolutionary Biology* **12**: 1036-1039.

Michiels, N.K., Beukeboom, L.W., Pongratz, N. and Zeitlinger, J. (2001) Parthenogenetic flatworms have more symbionts than their coexisting, sexual conspecifics, but does this support the Red Queen? *Journal of Evolutionary Biology* **14**: 110-119.

Mitchell, S.E. and Lampert, W. (2000) Temperature adaptation in a geographically widespread zooplankter, *Daphnia magna*. *Journal of Evolutionary Biology* **13**: 371-382.

Mitchell, S.E., Rogers, E.S., Little, T.J. and Read, A.F. (2005) Host-parasite and genotype-by-environment interactions: temperature modifies potential for selection by a sterilizing pathogen. *Evolution* **59**: 70-80.

Moret, Y. and Schmid-Hempel, P. (2001) Immune defence in bumble-bee offspring.

*Nature* **414**: 506-507.

Moret, Y. and Siva-Jothy, M.T. (2003) Adaptive innate immunity? Responsive-mode prophylaxis in the mealworm beetle, *Tenebrio molitor*. *Proceedings of the Royal Society of London, series B* **270**: 2475-2480.

Moritz, C., McCallum, H., Donnellan, S. and Roberts, J.D. (1991) Parasite loads in parthenogenetic and sexual lizards (*Heteronotia binoei*): support for the Red Queen hypothesis. *Proceedings of the Royal Society of London, series B* **244**: 145-149.

Mukai, T. (1964) The genetic structure of natural populations of *Drosophila melanogaster*. I. Spontaneous mutation rate of polygenes controlling viability. *Genetics* **50**: 1-19.

Mukai, T. (1969) The genetic structure of natural populations of *Drosophila melanogaster*. VII. Synergistic interaction of spontaneous mutant polygenes controlling viability. *Genetics* **61**: 749-761.

Mukai, T. (1970) Viability mutations induced by ethyl methanesulfonate in *Drosophila melanogaster*. *Genetics* **65**: 335-348.

Nolan, P.M., Peters, J., Vizer, L., Strivens, M., Washbourne, R., Hough, T., Wells, C., Glenister, P., Thornton, C., Martin, J., Fisher, E., Rogers, D., Hagan, J., Reavill, C., Gray, I., Wood, J., Spurr, N., Browne, M., Rastan, S., Hunter, J. and Brown, S.D.M. (2000) Implementation of a large-scale ENU mutagenesis program: towards increasing the mouse mutant resource. *Mammalian Genome* **11**: 500-506.

Nunn, C.L., Gittleman, J.L. and Antonovics, J. (2000) Promiscuity and the primate immune system. *Science* **290**: 1168-1170.

Ohnishi, O. (1977)a Spontaneous and ethyl methanesulfonate-induced mutations controlling viability in *Drosophila melanogaster*. I. Recessive lethal mutations. *Genetics* **87**: 519-527.

Ohnishi, O. (1977)b Spontaneous and ethyl methanesulfonate-induced mutations controlling viability in *Drosophila melanogaster*. II. Homozygous effect of polygenic mutations. *Genetics* **87**: 529-545.

Ohnishi, O. (1977)c Spontaneous and ethyl methanesulfonate-induced mutations controlling viability in *Drosophila melanogaster*. III. Heterozygous effect of polygenic mutations. *Genetics* **87**: 547-556.

Otto, S.P. and Feldman, M.W. (1997) Deleterious mutations, variable epistatic interactions, and the evolution of recombination. *Theoretical Population Biology* **51**: 134-147.

Otto, S.P. and Lenormand, T. (2002) Resolving the paradox of sex and recombination. *Nature Reviews Genetics* **3**: 252-261.

Otto, S.P. and Nuismer, S.L. (2004) Species interactions and the evolution of sex. *Science* **304**: 1018-1020.

Peck, J.R. (1994) A ruby in the rubbish: beneficial mutations and the evolution of sex. *Genetics* **137**: 597-606.

Peck, J.R. and Waxman, D. (2000) Mutation and sex in a competitive world. *Nature* **406**: 399-404.

Penn, D.J. and Potts, W.K. (1999) The evolution of mating preferences and major histocompatibility complex genes. *American Naturalist* **153**: 145-164.

Peters, A.D. (1999) The effects of pathogen infection and mutation on life-history characters in *Arabidopsis thaliana*. *Journal of Evolutionary Biology* **12**: 460-470.

Peters, A.D. and Keightley, P.D. (2000) A test for epistasis among induced mutations in *Caenorhabditis elegans*. *Genetics* **156**: 1635-1647.

Peters, A.D. and Lively, C.M. (1999) The Red Queen and fluctuating epistasis: a population genetic analysis of antagonistic coevolution. *American Naturalist* **154**: 393-405.

Rice, W.R. (1998) Requisite mutational load, pathway epistasis, and deterministic mutation accumulation in sexual versus asexual populations. *Genetica* **103**: 71-81.

Rice, W.R. (2002) Experimental tests of the adaptive significance of sexual recombination. *Nature Reviews Genetics* **3**: 241-251.

Riley, B.B. and Grunwald, D.J. (1995) Efficient induction of point mutations allowing recovery of specific locus mutations in zebrafish. *Proceedings of the National Academy of Sciences, USA* **92**: 5997-6001.

Riley, M.A. and Gordon, D.M. (1999) The ecological role of bacteriocins in bacterial competition. *Trends in Microbiology* **7**: 129-133.

Rivero, A., Balloux, F. and West, S.A. (2003) Testing for epistasis between deleterious mutations in a parasitoid wasp. *Evolution* **57**: 1698-1703.

Ruvinsky, A.O., Pereygin, A.A., Lobkov, Y.I. and Belyaev, D.K. (1986) Factors organising and maintaining polymorphism in a cyclic parthenogenetic species: *Daphnia pulex*. *Heredity* **57**: 15-22.

Salathé, P. and Ebert, D. (2003) The effects of parasitism and inbreeding on the competitive ability in *Daphnia magna*: evidence for synergistic epistasis. *Journal of Evolutionary Biology* **16**: 976-985.

Schartl, M., Nanda, I., Schlupp, I., Wilde, B., Epplen, J.T., Schmid, M. and Parzefall, J. (1995) Incorporation of subgenomic amounts of DNA as compensation for mutational load in a gynogenetic fish. *Nature* **373**: 68-71.

Schenck, R.A. and Vrijenhoek, R.C. (1986) Spatial and temporal factors affecting coexistence among sexual and clonal forms of *Poeciliopsis*. *Evolution* **40**: 1060-1070.

Schmid-Hempel, P. (2003) Variation in immune defence as a question of evolutionary ecology. *Proceedings of the Royal Society of London, series B* **270**: 357-366.

Schmid-Hempel, P. (2005) Natural insect host-parasite systems show immune-priming and specificity: puzzles to be solved. *BioEssays* **27**: 1026-1034.



Schmid-Hempel, P. and Funk, C.R. (2004) The distribution of genotypes of the trypanosome parasite, *Crithidia bombi*, in populations of its host, *Bombus terrestris*. *Parasitology* **129**: 147-158.

Schulenburg, H. and Ewbank, J.J. (2004) Diversity and specificity in the interaction between *Caenorhabditis elegans* and the pathogen *Serratia marcescens*. *BMC Evolutionary Biology* **4**: 49.

Schwartz, S.S. and Cameron, G.N. (1993) How do parasites cost their hosts – preliminary answers from trematodes and *Daphnia obtusa*. *Limnology and Oceanography* **38**: 602-612.

Shabalina, S.A., Yampolsky, L.Y. and Kondrashov, A.S. (1997) Rapid decline of fitness in panmictic populations of *Drosophila melanogaster* maintained under relaxed natural selection. *Proceedings of the National Academy of Sciences, USA* **94**: 13034-13039.

Siller, S. (2001) Sexual selection and the maintenance of sex. *Nature* **411**: 689-692.

Simon, J.-C., Delmotte, F., Rispe, C. and Crease, T. (2003) Phylogenetic relationships between parthenogens and their sexual relatives: the possible routes to parthenogenesis in animals. *Biological journal of the Linnaean Society* **79**: 151-163.

Smith, V.J., Brown, J.H. and Hauton, C. (2003) Immunostimulation in crustaceans: does it really protect against infection? *Fish & Shellfish Immunology* **15**: 71-90.

Sokal, R.R. and Rohlf, F.J. (1995) *Biometry (3rd edn.)*. W.H. Freeman and Co., New York.

Solnica-Krezel, L., Schier, A.F. and Driever, W. (1994) Efficient recovery of ENU-induced mutations from the zebrafish germline. *Genetics* **136**: 1401-1420.

Stacey, D.A., Thomas, M.B., Blanford, S., Pell, J.K., Pugh, C. and Fellowes, M.D.E. (2003) Genotype and temperature influence pea aphid resistance to a fungal entomopathogen. *Physiological Entomology* **28**: 75-81.

Stirnadel, H.A. and Ebert, D. (1997) Prevalence, host specificity, and impact on host fecundity of microparasites and epibionts in three sympatric *Daphnia* species. *Journal of Animal Ecology* **66**: 212-222.

Storhas, M., Weinzierl, R.P. and Michiels, N.K. (2000) Paternal sex in parthenogenetic planarians: a tool for investigating the accumulation of deleterious mutations. *Journal of Evolutionary Biology* **13**: 1-8.

Szafraniec, K., Borts, R.H. and Korona, R. (2001) Environmental stress and mutational load in diploid strains of the yeast *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences, USA* **98**: 1107-1112.

Thomas, M.B. and Blanford, S. (2003) Thermal biology in insect-parasite interactions. *Trends in Ecology and Evolution* **18**: 344-350.

Trivers, R.L. and Willard, D.E. (1973) Natural selection of parental ability to vary the sex ratio of offspring. *Science* **179**: 90-92.

Underwood, A.J. (1997) *Experiments in ecology: Their logical design and interpretation using analysis of variance*. Cambridge University Press, Cambridge.

Vassilieva, L.L., Hook, A.M. and Lynch, M. (2000) The fitness effects of spontaneous mutations in *Caenorhabditis elegans*. *Evolution* **54**: 1234-1246.

Vernon, J.G., Okamura, B., Jones, C.S. and Noble, L.R. (1996) Temporal patterns of clonality and parasitism in a population of freshwater bryozoans. *Proceedings of the Royal Society of London, Series B* **263**: 1313-1318.

Vrijenhoek, R.C. (1994) Unisexual fish: Model systems for studying ecology and evolution. *Annual Review of Ecology and Systematics* **25**: 71-96.

Watson, F.L., Püttmann-Holgado, R., Thomas, F., Lamar, D.L., Hughes, M., Kondo, M., Rebel, V.I. and Schmucker, D. (2005) Extensive diversity of Ig-superfamily proteins in the immune system of insects. *Science* **309**: 1874-1878.

Weider, L.J. (1993) A test of the "general-purpose" genotype hypothesis: differential tolerance to thermal and salinity stress among *Daphnia* clones. *Evolution* **47**: 965-969.

Weinzieri, R.P., Berthold, K., Beukeboom, L.W. and Michiels, N.K. (1998) Reduced male allocation in the parthenogenetic hermaphrodite *Dugesia polychroa*. *Evolution* **52**: 109-115.

West, S.A., Lively, C.M. and Read, A.F. (1999) A pluralist approach to sex and recombination. *Journal of Evolutionary Biology* **12**: 1003-1012.

West, S.A., Peters, A.D. and Barton, N.H. (1998) Testing for epistasis between deleterious mutations. *Genetics* **149**: 435-444.

Whitlock, M.C. and Bourguet, D. (2000) Factors affecting the genetic load in *Drosophila*: synergistic epistasis and correlations among fitness components. *Evolution* **54**: 1654-1660.

- Winsor, G.L. and Innes, D.J. (2002) Sexual reproduction in *Daphnia pulex* (Crustacea: Cladocera): observations on male mating behaviour and avoidance of inbreeding. *Freshwater Biology* **47**: 441-450.
- Wloch, D.M., Borts, R.H. and Korona, R. (2001) Epistatic interactions of spontaneous mutations in haploid strains of the yeast *Saccharomyces cerevisiae*. *Journal of Evolutionary Biology* **14**: 310-316.
- Wuethrich, B. (1998) Why sex? Putting theory to the test. *Science* **281**: 1980-1982.
- You, L. and Yin, J. (2002) Dependence of epistasis on environment and mutation severity as revealed by *in silico* mutagenesis of Phage T7. *Genetics* **160**: 1273-1281.
- Zhang, S-M., Adema, C.M., Kepler, T.B. and Loker, E.S. (2004) Diversification of Ig superfamily genes in an invertebrate. *Science* **305**: 251-254.